Kinetic Analysis of Nonphotochemical Quenching of Chlorophyll Fluorescence. 2. Isolated Light-Harvesting Complexes†

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ABSTRACT: The chlorophyll fluorescence yield of purified photosystem II light-harvesting complexes can be lowered by manipulation of experimental conditions. In several important respects, this quenching resembles the nonphotochemical quenching observed in isolated chloroplasts and leaves, therefore providing a model system for investigating the underlying mechanism. A methodology based on the principles of enzyme kinetic analysis has already been applied to isolated chloroplasts, and this same experimental approach was used here with purified LHCIIb, CP26, and CP29. It was found that the kinetics of the decrease in fluorescence yield robustly fitted a second-order kinetic model with respect to time after induction of quenching. The second-order rate constant was dependent upon the complex that was analyzed, the detergent concentration, the solution pH, and the presence of exogenous xanthophyll cycle carotenoids. In contrast, the formation of an absorbance change at 683 nm that accompanies quenching displayed first-order kinetics. The reversal of quenching also displayed second-order kinetics. These data show that quenching results from a binary reaction, possibly arising between two chlorophyll molecules. On the basis of these data, a model for the regulation of nonphotochemical quenching based upon the allosteric control of the conformation of light-harvesting complexes by protonation and xanthophyll binding is presented.

It has been demonstrated that the dissipation of excess excitation energy in the light-harvesting antenna of photosystem II, detected as the nonphotochemical quenching of chlorophyll fluorescence, can be described by a relatively simple kinetic model (*1*). In isolated chloroplasts, the response to the thylakoid lumen pH follows sigmoidal kinetics indicative of positive cooperativity of proton binding. Allosteric effectors such as zeaxanthin, antimycin, and dibucaine control the apparent K_m for protons and the degree of cooperativity. These effectors were also shown to control the rate of induction of quenching. Quenching was accelerated by the presence of zeaxanthin (as compared to violaxanthin) and by the addition of dibucaine, and inhibited by the addition of antimycin A. It was found that the decrease in fluorescence, initiated by illumination to generate a ΔpH,[1] followed second-order kinetics, and the allosteric effectors affected only the second-order rate constant. The kinetics were hyperbolic, clearly demonstrated by the linearity of the plot of the reciprocal of the fluorescence decrease versus time. The simplest explanation of the hyperbolic kinetics is that it arises from a binary reaction between two identical species (A + A → 2A). Since fluorescence was the measured parameter, this suggests that A is a Chl molecule bound to a PSII antenna protein, and that 2A is a species with a low fluorescence yield. In turn, this is consistent with a relatively simple model for the mechanism of nonphotochemical quenching in which energy dissipation may arise directly as a result of the formation of a Chl dimer, although other explanations are not excluded. Previous data obtained from whole leaves showed broadly similar kinetic features of fluorescence quenching as isolated chloroplasts (*2*).

To explore this model further, it is necessary to use a simpler system of isolated chlorophyll proteins. It has been shown that the LHCII components, LHCIIb, CP26, and CP29, each show fluorescence quenching upon manipulation of the in vitro conditions (*3*−*8*). There are a number of striking similarities between this quenching and the process of qE in vivo. Most notable is that in vitro quenching may be controlled by either the endogenous (*2*) or exogenous content (*3*, *4*, *7*, *8*) of xanthophyll cycle carotenoid. In addition, the absorbance changes associated with quenching in isolated LHCII are also found upon formation of qE (*9*, *10*). Finally, inhibitors (antimycin and DCCD) and enhancers (dibucaine) of qE (*1*, *11*) had the same effects on in vitro quenching (*3*). In the case of DCCD, this reagent binds only to Lhcb4 and Lhcb5 of the Lhcb proteins in vivo (*12*), only binds to these proteins in vitro (*12*), and inhibits in vitro quenching only in CP29 and CP26 (*6*). Therefore, quenching in isolated LHCII serves as a model system for understanding the mechanism of qE. This view has been strengthened by

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[1] Abbreviations: DCCD, dicyclohexylcarbodiimide; DM, *n*-dodecyl β-maltoside; Lhcb, proteins encoded by the *Lhcb* genes; LHCII, light-harvesting complexes of photosystem II; PSII, photosystem II; qE, nonphotochemical quenching dependent upon the ΔpH; ΔpH, transthylakoid pH gradient.

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the observation that pretreatment of leaves with light, to convert violaxanthin to zeaxanthin, causes an acceleration in the development of quenching in the leaves, in the chloroplasts isolated from those leaves, and in the LHCII purified from the chloroplasts (*2*).

In this paper, isolated LHCII has been used to examine the kinetic features of the formation of quenching. It is shown that quenching is a binary reaction that can occur within a single protein subunit, and that this reaction results in formation of a chlorophyll species absorbing at 683 nm.

MATERIALS AND METHODS

Light-harvesting complexes were purified from spinach leaves as previously described (*13*). Investigation of the quenching of chlorophyll fluorescence followed two previously well-documented protocols. In the first, samples were dissolved in 200 μM DM, preincubated with added modulators, and diluted into a detergent free medium at a specified pH to give a final DM concentration of 6 μM (unless stated otherwise); this dilution induced a process of spontaneous quenching (*3*, *4*). In the second, samples dissolved in 200 μM DM were incubated for 2 min and quenching was induced by addition of the modulator or lowering of the pH (*8*). Chlorophyll fluorescence yield was measured as previously described (*4*) and displayed on a chart recorder. Data points were obtained by digitization of chart recorder traces using Ungraph (Cambridge Soft). The absorption changes accompanying quenching were measured using an SLM DW2000 spectrophotometer as described previously (*4*). Kinetic analysis was carried out as described in the previous paper (*1*), and data were fitted to a simple hyperbolic decay:

$$F = 1/(kt + 1/F_q) + F_u \quad (1)$$

where k is the second-order rate constant, F_q is the amplitude of quenchable fluorescence, and F_u is the amplitude of unquenchable fluorescence.

RESULTS

Fluorescence quenching in isolated LHCIIb is pH-dependent (*2*). In Figure 1, the extent of quenching recorded 30 s after dilution into a reduced detergent medium at specified pH is presented. The extent of quenching can be titrated as a function of pH. The titration appears to arise from two waves, one between pH 7 and 5 and a second between pH 5 and 4. The latter has an approximate pK of 4.4 in the control. The pK of this phase can be shifted to higher pH, by either the reduction in DM concentration (Figure 1A), the addition of zeaxanthin (Figure 1B), or the presence of dibucaine (Figure 1C). The effects of the latter two are reminiscent of their effects on qE in isolated chloroplasts (*1*), except that in Figure 1 there is no evidence of cooperativity for H^+ binding. Similar behavior was displayed by both CP26 and CP29 (not shown).

The kinetics of quenching were also found to be similar to those displayed by qE in chloroplasts (Figure 2). As found previously (*4*), quenching is more rapid in CP26 and CP29 than in LHCIIb (Figure 2A). In each case, however, the quenching fitted a second-order reaction, as evidenced by the good fits to hyperbolic kinetics (Figure 2A) and linear reciprocal plots (Figure 2B), with both the rate constant and the maximum amount of quenching varying between complexes. CP29 displayed the highest second-order rate constant.

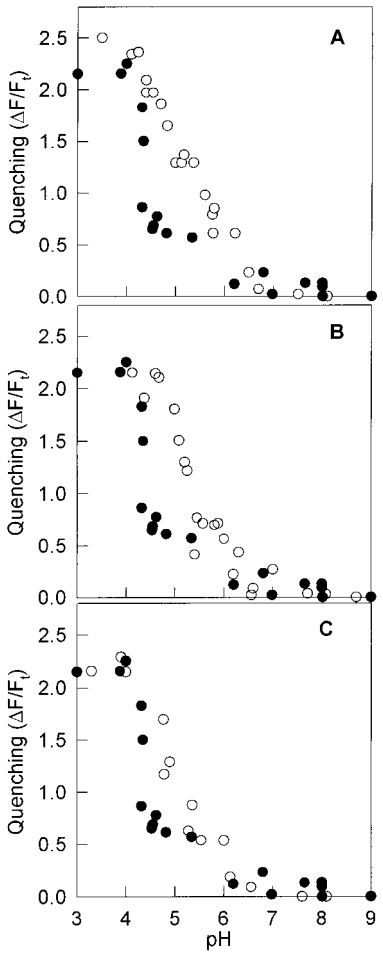

FIGURE 1: Quenching of chlorophyll fluorescence in isolated LHCIIb as a function of medium pH. (A) Effect of reducing the DM concentration to 6 μM, (B) effect of 20 μM zeaxanthin, and (C) effect of 100 μM dibucaine: control (●) (the same data are shown in each panel) and treatment (○). Quenching was initiated by dilution of the complex into a medium containing 60 μM DM at the specified pH as described in the text. Quenching was calculated as the change in fluorescence (ΔF) divided by the fluorescence intensity after 30 s (F_t).

External agents strongly affect the kinetics of quenching in the in vitro system (*3*, *4*, *7*). Figure 3 shows that in all cases, the same second-order kinetic model describes the fluorescence quenching data (Figure 3A), and linear reciprocal plots were obtained (Figure 3B). The second-order rate constant for quenching in CP26 can be reduced by addition of violaxanthin and DM, and increased by dibucaine and zeaxanthin. These modulators exert control over quenching in LHCIIb and CP29 as well as CP26 (Table 1). Inhibitors reduced the rate constant and the amplitude of quenching, the combined effect being a strong control over the quenching half-time. Most notable were the different effects of violaxanthin and zeaxanthin. In the presence of zeaxanthin compared to violaxanthin, the half-time decreased by factors of 11.7, 7.2, and 8.4 for CP26, CP29, and LHCIIb, respectively. Violaxanthin induced a strong increase in half-time compared to the control, by factors of 2.4, 2.0, and 3.8 for CP26, CP29, and LHCIIb, respectively.

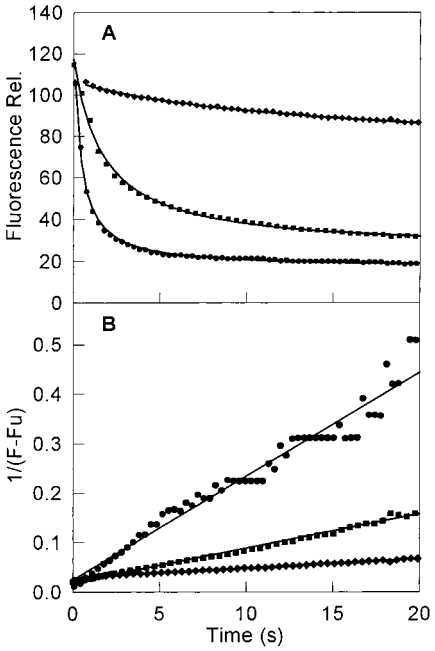

FIGURE 2: Kinetics of chlorophyll fluorescence quenching in isolated LHCII. (A) Decrease in fluorescence. Data points were obtained by digitization of chart recorder traces, and the line represents the best fit generated using eq 1. (B) Second-order reciprocal plot of the data, where F is the level of fluorescence and F_u is the unquenched fluorescence, with linear regressions giving r^2 values of 0.96−0.98: CP29 (●), CP26 (■), and LHCIIb (◆).

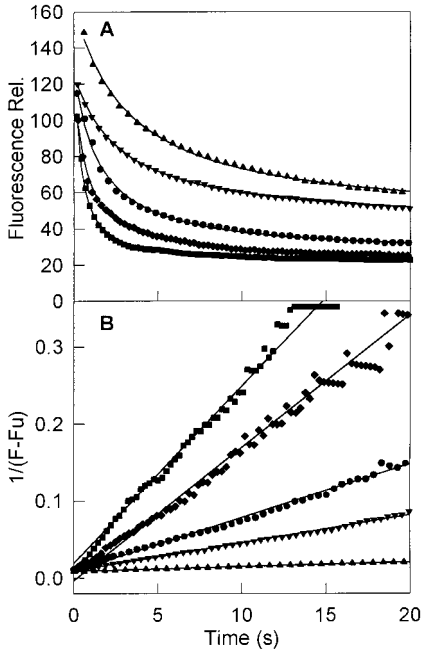

FIGURE 3: Kinetics of chlorophyll fluorescence quenching in isolated CP26 induced by dilution into 6 μM DM at pH 8.0. (A) Decrease in fluorescence. (B) Second-order reciprocal plot of the data (r^2 = 0.98−0.99). For other details, see the legend of Figure 2: control (●), 100 μM dibucaine (■), 60 μM DM (▲), 20 μM violaxanthin (▼), and 20 μM zeaxanthin (◆).

Recently, we showed that fluorescence quenching can be observed under conditions when the DM concentration is set to higher levels to prevent the spontaneous events that lead to protein aggregation (*8*). At this detergent concentration, there are no perturbations in pigment organization. The total amplitude of quenching is restricted to ~60% of maximum fluorescence in this system. To further test for the presence of an intrinsic ubiquitous fluorescence quenching process in the light-harvesting complexes, this system was analyzed for reaction order. Again, it was found that in every case quenching fitted second-order kinetics. In Figure 4, the kinetics of quenching induced by addition of the carotenoids antheraxanthin, zeaxanthin, and auroxanthin are analyzed. Antheraxanthin caused quenching with a low amplitude and a slow rate. The rate and amplitude were both higher upon addition of zeaxanthin, and were highest when quenching was induced by addition of auroxanthin. The differential effect of these carotenoids is predicted from earlier studies (*7*).

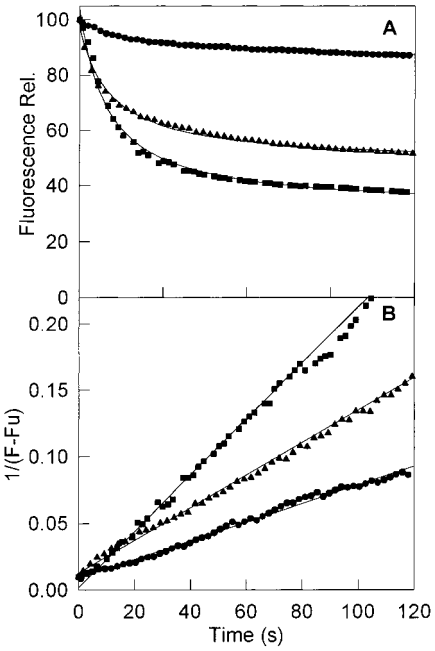

FIGURE 4: Kinetics of chlorophyll fluorescence quenching in isolated CP26 induced by addition of carotenoid in the presence of 200 μM DM. (A) Decrease in fluorescence. (B) Second-order reciprocal plot of the data. r^2 = 0.99 in each case. For other details, see the legend of Figure 2: 20 μM antheraxanthin (●), 20 μM zeaxanthin (▲), and 10 μM auroxanthin (■).

Quenching can also be induced in the presence of high DM concentrations by the addition of acid to lower the pH. Although the extent of quenching is lower than that found in the low-detergent "spontaneous" system (e.g., Figure 1), the kinetics were again the same: a good fit to second-order hyperbolic kinetics and a linear reciprocal plot (Figure 5).

The quenching process was further probed by analyzing the kinetics of reversal. It has previously been shown that quenching in isolated LHCII can be reversed by addition of detergent (*3*) or DCCD (*6*) or increasing the pH (M. Wentworth, unpublished data). It was found that the reversal was hyperbolic and fitted second-order kinetics (Figure 6).

It has been found that the in vitro quenching process is associated with very characteristic changes in the visible absorption spectrum of the complex (*9*, *10*). Ubiquitous is a change in the chlorophyll red band, with a positive change at 683 nm. This absorption change was found to correlate with fluorescence quenching in a quasi-linear manner (*8*), and it was therefore suggested that the species responsible is tightly linked to the quencher, or may be the quencher itself (*8*). The analysis of the kinetics of the 683 nm change

Table 1: Quenching Parameters for CP29, CP26, and LHCIIb[a]

complex	treatment	k ($\times 10^3$ s^{-1})	F_q	F_u	$\tau_{1/2}$ (s)	qN_o
LHCIIb	control	0.78 ± 0.06	29 ± 0.3	71 ± 2.0	45.2 ± 3.8	0.29 ± 0.006
	DM	0	0	99 ± 0.6	∞	0
	DB	0.11 ± 0.04	54.7 ± 0.9	45.4 ± 0.9	16.8 ± 0.8	0.55 ± 0.009
	Vio	0.36 ± 0.04	16.9 ± 0.1	83.2 ± 0.7	171.5 ± 24.4	0.17 ± 0.001
	Zea	1.20 ± 0.20	39.1 ± 1.9	59.3 ± 1.7	20.3 ± 1.4	0.40 ± 0.02
CP29	control	20	69.9 ± 1.1	30.0 ± 1.1	0.72 ± 0.01	0.70 ± 0.01
	DM	12	61.4 ± 1.9	37.2 ± 3.4	1.43 ± 0.09	0.62 ± 0.03
	DB	64 ± 6	79.9 ± 1.7	20.3 ± 1.5	0.20 ± 0.02	0.80 ± 0.02
	Vio	14	51.9 ± 1.3	47.9 ± 1.1	1.43 ± 0.07	0.52 ± 0.01
	Zea	66 ± 4	79.0 ± 0.4	20.8 ± 0.5	0.20 ± 0.01	0.79 ± 0.005
CP26	control	7 ± 1.2	75.1 ± 2.3	24.9 ± 2.2	1.99 ± 0.31	0.75 ± 0.02
	DM	3.4 ± 0.4	47.8 ± 2.6	51.1 ± 2.6	6.65 ± 1.35	0.48 ± 0.03
	DB	36 ± 4	86.2 ± 0.2	13.9 ± 0.3	0.32 ± 0.08	0.86 ± 0.003
	Vio	4	68.5	33.0 ± 0.4	4.70 ± 0.11	0.68 ± 0.002
	Zea	30 ± 6	82.5 ± 0.4	16.9 ± 0.7	0.40 ± 0.03	0.83 ± 0.01

[a] $\tau_{1/2}$, quenching half-time calculated as $1/(F_q k)$; qN_o, maximum potential quenching calculated as $F_q/(F_u + F_q)$; DM, 60 μM DM; DB, 100 μM dibucaine; Vio, 20 μM violaxanthin; Zea, 20 μM zeaxanthin. Errors represent the standard error from the mean and were calculated from at least four independent quenching curves. Where no errors are given, exactly the same value was obtained for each replicate.

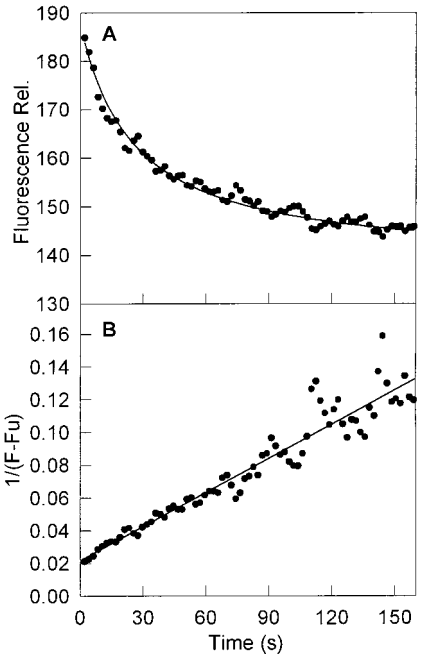

FIGURE 5: Kinetics of chlorophyll fluorescence quenching in isolated CP29 induced by addition of HCl to lower the pH to 4.0 in the presence of 200 μM DM. (A) Decrease in fluorescence. (B) Second-order reciprocal plot of the data ($r^2 = 0.92$). For other details, see the legend of Figure 2.

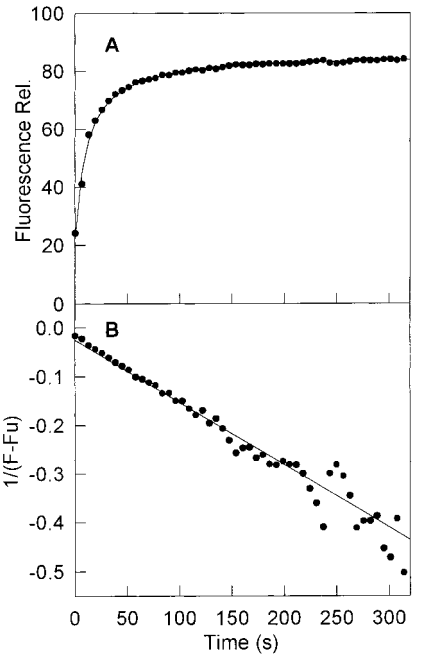

FIGURE 6: Kinetics of the reversal of chlorophyll fluorescence quenching in isolated CP26 induced by addition of 200 μM DM. Quenching was brought about by dilution into 6 μM DM as described in the legend of Figure 2. (A) Increase in fluorescence. (B) Second-order reciprocal plot of the data ($r^2 = 0.95$). For other details, see the legend of Figure 2.

induced by addition of zeaxanthin to CP26 is shown in Figure 7. The absorption change appeared to fit exponential rather than hyperbolic kinetics (Figure 7A). This was confirmed by the linearity of the semilogarithmic plot (Figure 7B) and the clear nonlinearity of the reciprocal plot (Figure 7C). Therefore, the formation of the 683 nm band appears to follow first-order kinetics.

DISCUSSION

An analysis of the kinetics of fluorescence quenching in isolated light-harvesting complexes has allowed a refinement of the proposals that have been made for the mechanism of nonphotochemical quenching. In confirmation of numerous previous reports (reviewed in refs *9* and *10*), it has been demonstrated here that many of the features of qE observed in isolated chloroplasts and leaves can be duplicated in isolated complexes. In particular, we now add a quantitative dimension to the previous observations (*3*, *4*, *6*−*8*) that the modulators of qE in vivo also control quenching in vitro. Particularly impressive is the differential behavior of violaxanthin and zeaxanthin in their control of both the apparent p*K* and the rate constant for in vitro quenching. It should be pointed out that the quenching described here is induced at very low irradiance (the measuring beam intensity of the fluorimeter, approximately 1 μmol m^{-2} s^{-1}). Here the in vivo properties of the excess light environment (violaxanthin deepoxidation, ΔpH) are simulated by an alteration in conditions. The system is different from the fluorescence quenching that is induced in isolated LHCII upon exposure to high irradiance (*14*), a phenomenon which appears to result from a direct effect of light on the organization of protein

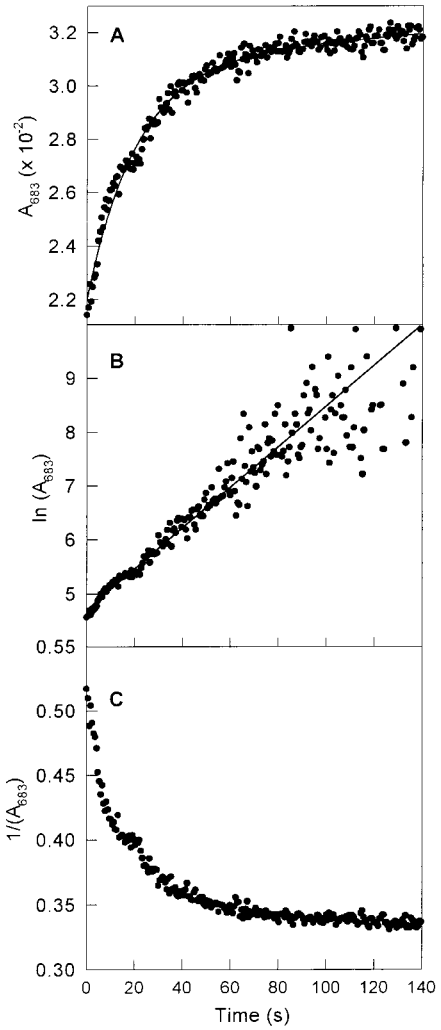

FIGURE 7: Kinetics of the absorbance change at 683 nm in isolated CP26 induced by addition of 20 μM zeaxanthin in the presence of 200 μM DM. (A) Increase in absorption with the data fitted to a first-order rate equation. (B) A semilogarithmic plot of the data, with a linear regression of the data ($r^2 = 0.90$). (C) A reciprocal second-order plot.

aggregates (*15*, *16*). However, there is a common feature in that structural changes in LHCII seem to underlie both quenching processes, and therefore, it is still possible that the quenching mechanism is the same even though the driving forces for the structural changes are different (*17*).

The kinetics of quenching in vitro robustly fit a single-component second-order reaction, not only for each of the three complexes tested but also in the presence of each modulator, and using two different quenching protocols. A plot of the reciprocal of fluorescence versus time in every case gave a linear relationship, and the effect of the modulator could be described only in terms of its effect on the second-order rate constant. Exactly the same conclusions arose from a kinetic analysis of qE in isolated chloroplasts (*1*), and even in leaves (*2*). The demonstration of second-order kinetics in isolated LHCII indicates that the same kinetics observed in more complex in vivo systems do not arise from artifact (e.g., from sample heterogeneity). It is also important that the kinetics can be observed in dilute solubilized LHCII dissolved in detergent above the critical micelle concentration when no artifacts from sample aggregation could arise.

The ubiquitous occurrence of second-order kinetics for quenching suggests that in all cases the molecular mechanism is of the same type. Hyperbolic reaction kinetics indicate a binary reaction between two identical or equivalent reactants, of the type A + A → 2A. In this case, if the concentration of A is measured it disappears with strictly hyperbolic kinetics. In these experiments, it is chlorophyll fluorescence that is disappearing with these kinetics, showing that A is a fluorescing species, i.e., a chlorophyll molecule. The product of the reaction, 2A, is weakly fluorescent or nonfluorescent. If A is a chlorophyll molecule, then 2A may be a dimer of chlorophyll. The kinetic model predicts that if 2A were measured, its rate of formation would follow first-order kinetics. It was found that an absorbance change at 683 nm follows first-order kinetics. This absorbance change has been found in all cases of in vitro quenching (*6*, *8*); such changes are much more difficult to record in vivo, but a similar qE-related absorbance change has been observed in isolated chloroplasts (*18*). The origin of the 683 nm change cannot be ascertained, but formation of chlorophyll dimers in various solvents or in liposomes has been shown to form such red-shifted species (*19*). In some cases, these are quenchers. Thus, the simplest explanation of our data is that quenching results from chlorophyll dimerization, explaining how the disappearance of fluorescence is second-order but formation of the quencher is first-order.

It has been shown that when quenching is induced at relatively high detergent concentrations in purified CP26 and CP29, no protein oligomerization is detectable, strongly suggesting that quenching occurs within a single LHCII subunit (*8*). Therefore, the binary reaction causing quenching occurs within a single complex, and this suggests that it involves the reaction between two bound chlorophyll molecules which give rise to the 683 nm dimeric species.

However, it should be considered that the data can be explained in other alternative ways. Rather than being a single chlorophyll molecule, "A" may be near identical, but independent, fluorescing domains of a light-harvesting complex. These two domains could be quenched in a concerted and identical manner, by a change in protein structure or by the presence of a carotenoid simultaneously quenching both of them. Thus, the data do not rule out previous suggestions of a role for zeaxanthin (or lutein) in Chl to carotenoid energy transfer (*20*) or catalyzing the internal conversion of Chl excited states (*21*). The 683 nm change may in turn represent an absorption shift arising from such changes in the pigment environment and/or interactions in the complex, rather than being the quencher itself. The fact that the 683 nm change is accompanied by absorbance changes in the 400−500 nm region (*4*, *8*), in part from carotenoids, indeed supports the idea that the formation of the quencher involves not only chlorophylls.

It also has to be considered whether the second-order kinetics arise not from the quenching reaction itself but from another reaction which is rate-limiting. For example, there may be a consecutive reaction (X → Y → Z) in which the first reaction is slow and second-order (for example, by virtue of the binding of two protons) and the second is a fast (first-order) reaction responsible for quenching. While such an explanation cannot be eliminated entirely, the fact that the reversal of quenching (Z → Y) is also second-order would argue strongly against it.

The observed rate of reaction, expressed as the half-time for quenching, is dependent upon the second-order rate constant and the amplitude of quenchable fluorescence. The rate constant reflects the probability of the reaction between A and A, whereas the amplitude is determined by how much A is available for reaction. For the isolated complexes, we found that for zeaxanthin-induced quenching both the amplitude and the rate constant depend on the zeaxanthin concentration. Thus, zeaxanthin determines how many complexes are capable of quenching, and how rapidly the quenching develops.

For qE, we have shown that the second-order rate constant depends on a number of factors: the ΔpH, the xanthophyll cycle de-epoxidation state, and the presence of inhibitors and enhancers. The rate of quenching is also dependent both upon the plant species (*22*) and upon the growth irradiance (*23*). Analysis of the composition of discrete fluorescence lifetime components during qE formation gave evidence of a state induced by ΔpH alone (*24*, *25*). Such a state could be equivalent to the activation of A, making A available for reaction. However, our data also indicate that the extent of de-epoxidation of violaxanthin to zeaxanthin also determines the amplitude of qE when the ΔpH is subsaturating. Therefore, in all respects, both protonation and de-epoxidation exert the same effect on the availability of A for reaction and determine the rate constant of the $A + A \rightarrow 2A$ reaction.

It is important to distinguish between two aspects of the molecular process that underlie qE formation: first, those which occur within an individual protein subunit, and second, those involving interaction between subunits. The former give rise to the intrinsic second-order quenching process, whereas the latter explain the cooperative kinetics. Evidence of cooperativity in the titration curves for quenching was not found in isolated complexes (Figure 1), but was clear for qE in chloroplasts (*1*). We may postulate that the first step is the protonation of the protein subunit, a process which is an absolute prerequisite for quenching. This protonation reaction is enhanced by the de-epoxidation of violaxanthin to zeaxanthin. This state may be equivalent to the pH-dependent state of the PSII antenna identified by Gilmore et al. (*24*, *25*). The subsequent second-order reaction is caused by a second protonation, again enhanced by the presence of zeaxanthin, and generates the 683 nm absorbance change. In the most general terms, we postulate that zeaxanthin enhances the structural flexibility of the pigment−protein complex, in line with previous observations (*5*). In terms of molecular detail, there may be an involvement of pH-dependent zeaxanthin binding (*25*), displacement of Chl from binding sites (*26*), or pH-dependent release of Ca^{2+} (*27*) in these events. This simple scheme explains how both the pH and the presence of zeaxanthin may enhance the amplitude and rate constant for quenching in the isolated LHCII components.

In vivo, we suggest that the sigmoidal kinetics and cooperativity arise from the molecular interactions between the protein subunits of the PSII antenna. Possibly, these involve the macro-organization of the thylakoid membrane, including interaction both in the plane of the membrane (packing) and between membranes (stacking) (*28*). It is suggested that the stacking process confers on the antenna structure a requirement for symmetry, and that the conformational change associated with quenching has to occur in connected subunits in a concerted manner. Thus, the macro-organization of the antenna establishes cooperative behavior by preventing the existence of mixed populations of the two different conformation states of the protein subunits according to the classical Monod−Wyman−Changeaux model. This interaction could be weakened by forces which enhance the packing process (e.g., the presence of zeaxanthin), or weaken the stacking forces (e.g., the reduction in LHCIIb content). In both cases, there is a decline in cooperativity of the pH dependence of qE formation (*1*, *29*). It is perhaps this change in macrostructure of the thylakoid that is necessary for the 535 nm absorbance change that correlates with qE (*30*, *31*). Similarly, the disruption of the PSII macrodomain, as detected by the disappearance of the ψ-type CD (*32*), is evidence of changes in membrane structure accompanying qE formation.

It has been found that qE is eliminated in the *npq4* mutant of *Arabidopsis* which is deficient in the PsbS protein (*33*). In terms of the data presented here, and in the accompanying paper, it is necessary to consider the role of this protein. The role of PsbS has been explained in two ways (*33*). First, PsbS could be the unique site of fluorescence quenching, having the binding sites for both protons and zeaxanthin. In this regard, fluorescence quenching could be a general property of the proteins encoded by *Lhc* and *Lhc*-related genes (*34*), with PsbS being the only protein that expresses such behavior in vivo. However, the involvement of just one protein seems to be inconsistent with the cooperative kinetics observed in thylakoids (*1*), and with the fact that xanthophyll cycle carotenoids are bound to all the LHCII components (*13*, *35*, *36*). Instead, perhaps PsbS is a proton and/or zeaxanthin binding site that drives the conformational transition and the quenching process in a neighboring LHCII.

Of particular interest is the loss of the 535 nm change in this mutant (*33*), an observation which indicates that the absence of PsbS in some way interferes with the conformational changes that are obligatorily linked to qE. It is important to note that there is a residual NPQ in this mutant, but it is slow to form and slow to relax (*17*, *33*). It has been shown that this quenching is pH-dependent and is accompanied by absorbance changes, but these are smaller and spectrally shifted compared to those of the wild type (*17*). The quenching is also independent of the de-epoxidation state of the xanthophyll cycle pool. It seems that the structural flexibility of the PSII antenna has been reduced, drastically decreasing both the amplitude and the rate constant for quenching. Therefore, an alternative explanation of the role of PsbS is that it has a key role in regulating the thylakoid macro-organization.

Clearly, more work is needed to understand how PsbS participates in the kinetic model we have presented for qE. Despite this uncertainty, it is important to emphasize again the physiological implications of this model (*37*). The allosteric model and the activation−inhibition roles played by the xanthophyll cycle carotenoids allow for very precise control of the PSII quantum yield and electron transport capacity. In limiting light, a sufficiently high ΔpH can be maintained for ATP synthesis without sacrificing quantum yield through unwanted qE, yet in high light, because of activation by zeaxanthin formation, qE can be fully engaged without the requirement for an excessive ΔpH, which would inhibit electron transport at the site of plastoquinol oxidation

or the donor side of PSII. Another intriguing aspect of qE is how the thylakoid engages just the right amount of quenching such that PSII is not over- or under-regulated. Our second-order model, in line with the earlier suggestions of Weis and Berry (*38*), Walters and Horton (*39*), and Gilmore et al. (*24*, *25*), indicates that qE does not arise by a dynamic Stern−Volmer type of quenching acting randomly in the pigment bed of PSII, but that separate antenna units become switched off by conversion to the quenched state. Such a switching mechanism may allow fine control over PSII electron transport.

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