Kinetic Analysis of Nonphotochemical Quenching of Chlorophyll Fluorescence. 1. Isolated Chloroplasts†

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ABSTRACT: Nonphotochemical quenching of chlorophyll fluorescence in plants is indicative of a process that dissipates excess excitation energy from the light-harvesting antenna of photosystem II. The major fraction of quenching is obligatorily dependent upon the thylakoid ΔpH and is regulated by the de-epoxidation state of the xanthophyll cycle carotenoids associated with the light-harvesting complexes. Basic principles of enzyme kinetics have been used to investigate this process in isolated chloroplasts. The extent of quenching was titrated against the estimated thylakoid lumen pH, and a sigmoidal relationship was obtained with a Hill coefficient of 4.5 and a p*K* of 4.7. Upon de-epoxidation, these parameters changed to 1.6 and 5.7, respectively. Antimycin A suppressed quenching, increasing the Hill coefficient and reducing the p*K*. The rate of induction of quenching fitted second-order kinetics with respect to illumination time, and the rate constant was dependent upon the ΔpH, the de-epoxidation state, the presence of antimycin, and also the presence of dibucaine, a quenching enhancer. All these data are consistent with the notion that quenching is caused by a conformational transition in a chloroplast thylakoid protein; this transition shows cooperativity with respect to proton binding, and is controlled by de-epoxidation state and various exogenous reagents.

Nonphotochemical quenching of chlorophyll fluorescence is indicative of an increase in the level of nonradiative energy dissipation in the light-harvesting antenna of photosystem II in plants. The major fraction of nonphotochemical quenching is obligatorily dependent upon the presence of the transthylakoid ΔpH[1] and is called qE (*1*). In addition to the dependence upon the ΔpH, qE is also correlated to the de-epoxidation of the carotenoid violaxanthin into zeaxanthin via the xanthophyll cycle (*2*). The importance of qE results from the fact that it shows that the level of excitation energy in the PSII antenna is regulated, and this is thought to prevent over-reduction of the electron-transfer chain and to provide protection from photodamage (*3*).

The site of qE is the antenna system of photosystem II (*3*), which comprises the CP47/43, the LHCII proteins, and a number of LHC-related proteins such as PsbS and ELIPs (*4*, *5*). LHCII consists of the products of six different genes, *Lhcb1*−*6*, organized into four different classes of Chl *a*−Chl *b*−xanthophyll protein complexes. Approximately 60% of the PSII Chl is bound by the major complex LHCIIb, a heterotrimer of the Lhcb1−3 proteins. Three other monomeric "minor" complexes (CP29, CP26, and CP24) together bind an additional ∼10% of the Chl.

The site of qE within the PSII antenna is less certain. There is evidence that the minor complexes, especially CP26 and CP29, are the sites of qE; they provide the binding sites for the qE inhibitor dicyclohexylcarbodiimide (*6*) and are reported to be enriched in the xanthophyll cycle carotenoid (*7*, *8*), and in one study, qE could be found in a plant containing Lhcb5 as its only Lhcb protein (*9*). More recently, it has been found that a mutant lacking the PsbS protein is deficient in qE, implicating this protein as the unique site of quenching (*10*). However, a component of quenching is retained in this mutant, and although kinetically it differs from qE in the wild-type plant, it shows some features such as pH dependency and association with absorbance changes around 500 nm that indicate that qE may be modified rather than eliminated (*11*).

The mode of action of zeaxanthin in qE formation has not been resolved. This carotenoid could either act directly as the quencher of Chl excited states or act indirectly by controlling the extent of a quenching process intrinsic to the complex (*12*). In the first, protonation is suggested to promote zeaxanthin binding (*13*), or a conformational change that brings zeaxanthin into the proximity of a Chl (*14*). In the second, protonation would bring about the conformational change required for quenching, and zeaxanthin is a regulator of this event (*3*, *15*). Although these are very different mechanisms, the common feature is the presence of an antenna protein(s) in two conformational states: an unprotonated unquenched state and a protonated quenched state. The correlation between qE and the absorption change at

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[1] Abbreviations: Lhcb, proteins encoded by the *Lhcb* genes; LHCII, light-harvesting complexes of photosystem II; qN, nonphotochemical quenching; PSII, photosystem II; qE, rapidly reversible nonphotochemical quenching dependent upon the ΔpH; ΔpH, transthylakoid pH gradient.

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535 nm (*16*−*18*) is evidence for the involvement of a change in conformation. Evidence for two states of the PSII antenna has been obtained from modeling of the quantitative features of qE (*19*) and, more directly, by the observation of a transition to a new distinct excited state lifetime upon formation of qE (*20*).

One approach to gaining new insights into the mechanisms of control of qE by the ΔpH and the xanthophyll cycle is to carry out experiments based upon the established principles of chemical kinetics, i.e., (a) to examine the level of qE (equivalent to reaction velocity) as a function of proton and zeaxanthin concentration and (b) to analyze the reaction rates as a function of these factors.

Some experiments of the former type have been carried out using isolated chloroplasts (*21*, *22*). Evidence of the interaction between ΔpH and zeaxanthin content was obtained, but interpreted in different ways; either protonation was required for zeaxanthin binding (*13*), or zeaxanthin binding increased the affinity of binding of protons (*21*). In both cases, the notion that qE is controlled allosterically was established. A common feature of allosterically regulated proteins is that they display a sigmoidal ligand binding curve. For qE, there are some indications of sigmoidicity with respect to the estimated lumen pH (*15*), but this was not rigorously established. Data obtained with green algae (*23*) and also indirect estimates of ΔpH in leaves also suggested that proton binding may exhibit sigmoidal kinetics (*24*).

There has been surprisingly little attention paid to the rate at which qE forms following illumination. It is well-established that qE forms much more slowly than the rate of change of the lumen pH, indicating that quenching is limited by the molecular changes that follow protonation. Nevertheless, it seems that qE forms more rapidly at high irradiance, and it is widely accepted that qE is controlled by the higher ΔpH occurring at higher irradiance. However, following measurements of the irradiance dependency of quenching in isolated LHCII and thylakoids, it has been suggested that a component of qE may occur as a direct result of illumination (*25*, *26*), providing an alternative explanation for the increase in the quenching rate at higher irradiance. A second important observation is that the rate of qE formation is increased if zeaxanthin is present at the start of illumination. This may occur upon re-illumination after a relatively short dark period (*27*), or in mutant plants which lack the zeaxanthin epoxidase and so have a constitutively high level of this xanthophyll (*28*). Recently, it was shown that both the rate and amplitude of quenching were increased in the presence of zeaxanthin, compared to those in its absence (*27*). Thus, it appears that the two factors which control the level of qE also determine the rate of its formation. An important feature to emerge from this work was that the rate of formation appeared to follow second-order kinetics (*27*), a surprising result with important implications for the mechanisms of quenching.

The purpose of the experiments described here was twofold. First, we established the extent of cooperativity involved in qE formation. Second, we extended and developed our earlier kinetic approach by carrying out a detailed quantitative treatment of fluorescence quenching kinetics in isolated chloroplasts, and determined the nature of the irradiance dependency of these kinetics. We conclude that our data can only fit a kinetic model, which involves highly cooperative interaction between proton binding sites, controlled by various allosteric effectors. Further, we show that the quenching process is a specific type of binary reaction that displays hyperbolic reaction kinetics under all conditions. The second-order rate constant for quencher formation is determined by the ΔpH and the presence of allosteric effector molecules. These data allow us to make important conclusions about the molecular processes underlying qE formation.

MATERIALS AND METHODS

Intact spinach chloroplasts were isolated using a method previously described (*21*). The methodology for recording qE, and estimating the ΔpH using 9-aminoacridine (9-aa) fluorescence quenching, has been described previously (*21*). Kinetic analysis was carried out using F_m' values obtained at different time intervals for a large number of samples to ensure accumulation of a sufficient number of data points. Sigmaplot software was used to fit the data to analytical models as described in ref *29*. The data in which qE is titrated against quenching of 9-aminoacridine were fitted to a curve defined by the equation

$$qN = qN_{max}Q^n/(Q^n + Q^n{}_0) \quad (1)$$

where qN_{max} is the theoretical maximum qN, Q is the level of 9-aminoacridine quenching defined as (unquenched − quenched)/unquenched, Q_0 is the level of 9-aminoacridine quenching corresponding to the ΔpH level at which $qN = 0.5qN_{max}$, and n is the cooperativity coefficient (Hill coefficient). qN was calculated as $(F_m - F_m')/F_m$. Under the conditions that were used, more than 90% of the qN was rapidly reversible and was ascribed to qE.

ΔpH data for corresponding values of 9-aminoacridine quenching were calculated using the equation

$$\Delta pH = \log[1/(1 - Q) + Q/(1 - Q)](V_{out}/V_{in}) \quad (2)$$

where Q is the level of 9-aminoacridine quenching, V_{out} is the sample volume (1 mL), and V_{in} is the lumen volume, calculated for the sample chlorophyll concentration of 35 μg Chl/mL using the relationship $V_{in} = 50$ L/mol (*30*).

RESULTS

Figure 1 shows the results of a titration of qE versus ΔpH in chloroplasts isolated from dark-adapted and light-treated plants. The effect of the presence of zeaxanthin is to shift the ΔpH requirement to lower values. This has been reported many times (*11*, *21*, *31*). Also shown in Figure 1 is the effect of antimycin A, a well-known inhibitor of qE (*32*). The effect of this reagent is to shift the ΔpH requirement to higher values. In dark-adapted chloroplasts, this shift is sufficient for almost complete elimination of qE; only the first part of the titration was observed, up to the point of the maximum ΔpH that can be sustained by the thylakoid membranes. In light-activated chloroplasts, the full titration with antimycin was observed. Thus, the apparent pK for qE formation is dependent upon not only the de-epoxidation state but also the presence of a qE inhibitor. The antagonism between de-epoxidation state and antimycin A is evidence for an allosteric model for qE, where zeaxanthin is a positive effector and antimycin A a negative effector.

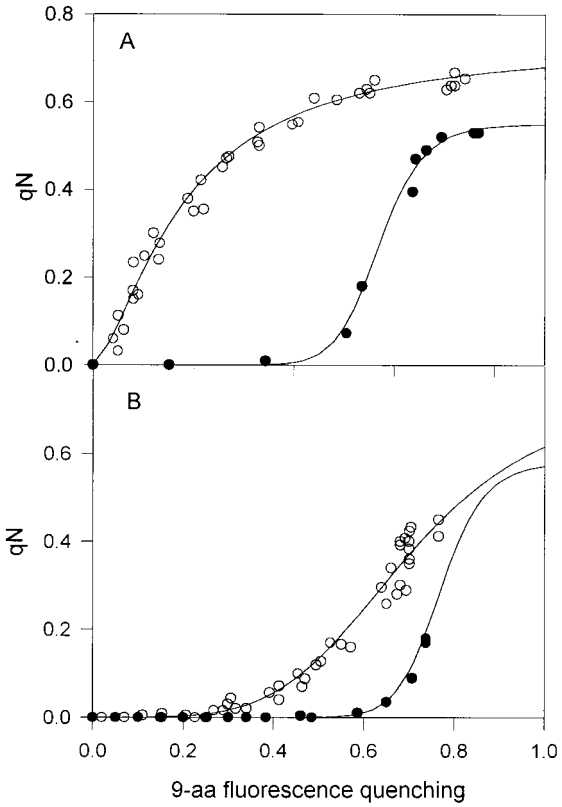

FIGURE 1: Relationship between qN and ΔpH, measured by the level of 9-aminoacridine fluorescence quenching. Data were obtained using different light intensities to vary ΔpH and, for each plot, were obtained from at least three different chloroplast preparations. (A) Chloroplasts from leaves pre-illuminated to induce zeaxanthin formation. (B) Chloroplasts containing violaxanthin only. Control (○) and in the presence of 100 nM antimycin A (●). All quenching was rapidly reversible and therefore of the qE type. Solid lines represent theoretical fits obtained using eq 1.

Table 1: Parameters Calculated from the qE vs 9-aa Titration Curves[a]

parameter	control (with zeaxanthin)	with zeaxanthin and antimycin	control (without zeaxanthin)	without zeaxanthin and with antimycin
n	1.6	13	4.5	16
p*K*	5.7	4.9	4.7	4.3
qN_{max}	0.75	0.71	0.74	0.58

[a] Parameters were obtained from the data in Figure 1. Equation 1 was used to calculate the Hill coefficient of cooperativity (*n*). The p*K* was calculated after transforming the 9-aminoacridine quenching data into pH as described in eq 2, and estimated as the lumen pH giving half-maximum quenching. qN_{max} is the maximum level of quenching estimated from the fitting of eq 1. Although the data are single values, they were obtained by fitting to data obtained from several separate experiments.

A particularly striking feature of Figure 1 is that, in addition to the shifts in the apparent p*K* for qE formation, each of the titration curves showed very different extents of sigmoidicity. The extent of sigmoidicity can be quantified by transforming the data with the Hill equation (eq 1). The Hill coefficients are shown in Table 1. A wide range of values was obtained, ranging from 16 in dark-adapted chloroplasts with antimycin to 1.6 in chloroplasts containing zeaxanthin. In each case, a shift in p*K* was associated with a change in sigmoidicity, and the lower the p*K*, the higher the cooperativity.

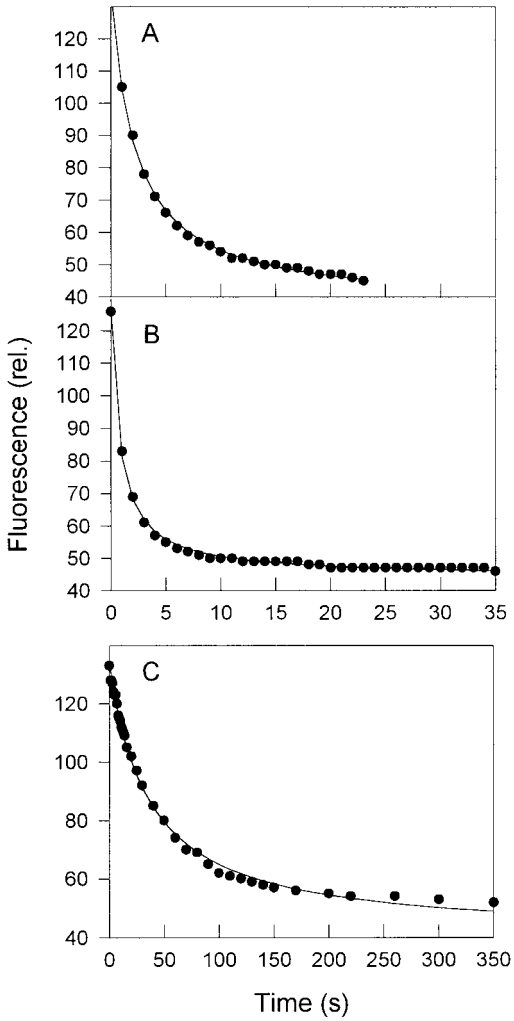

FIGURE 2: Effects of dibucaine and antimycin A on the nonphotochemical fluorescence quenching kinetics in intact chloroplasts isolated form light-treated leaves containing zeaxanthin: (A) control, (B) with 40 μM dibucaine, and (C) with 10 nM antimycin A. The light intensity was 1200 μM m^{-2} s^{-1}.

The rate of formation of the quenched state reveals information about the mechanism of control of qE and also the molecular mechanism involved in quenching. We have previously shown that the rate of qE formation in chloroplasts is dependent upon the de-epoxidation of violaxanthin (*27*). Here we have analyzed the reaction rate as a function of a range of factors controlling the extent of qE.

The rate of formation of qE can be varied by known quenching modulators, dibucaine and antimycin A (Figure 2). In this experiment, the conditions were adjusted so that the maximum extent of qE was rather similar with and without antimycin. The half-time for qE formation was 35 s in the presence of antimycin compared to the control value of 2.5 s. Dibucaine has been reported to enhance qE formation (*18*). It was found that this reagent caused an acceleration of qE formation, the half-time decreasing to 0.9 s.

The fluorescence quenching data have been found to fit a second-order equation of the type (*27*)

$$F = 1/(kt + 1/F_q) + F_u \quad (3)$$

where *k* is the second-order rate constant, F_q is the amplitude

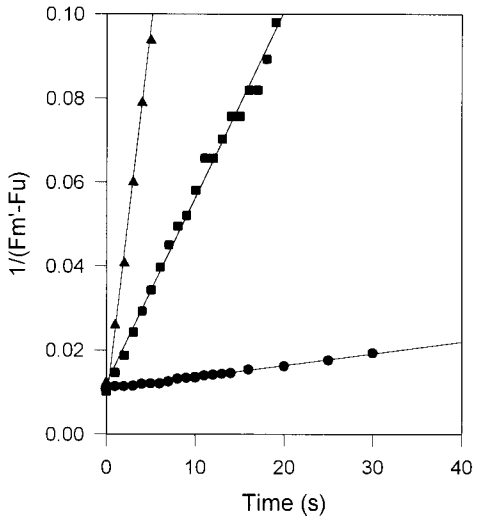

FIGURE 3: Reciprocal plots of the fluorescence quenching curves from Figure 2: control (■), with dibucaine (▲), and with antimycin A (●).

of the quenchable fluorescence ($F_m - F_u$), F_m is the maximum yield of fluorescence in the absence of ΔpH, and F_u is the amplitude of unquenchable fluorescence, which is constant throughout of the whole quenching period. This equation fitted well the quenching profiles shown in Figure 2. Using this equation, the theoretical limiting value of total nonphotochemical quenching, qN (qNo), equals ($F_m - F_u$)/F_m. The reciprocal, 1/($F_m' - F_u$), of the fluorescence quenching kinetics shown in Figure 2 is plotted versus time (Figure 3), where F_m' is the maximum yield of fluorescence in a quenched state. This enabled the differences between the kinetics to be seen more clearly and, most importantly, to show that in all cases the reciprocal transformation gave a linear relationship between time and fluorescence. Since a hyperbolic function gives a straight line upon reciprocal transformation, the use of eq 3 to fit the experimental data is firmly justified. It can be calculated from Figure 3 that the second-order rate constants are dramatically different between the control (5×10^{-3} s^{-1}), that with antimycin (0.28×10^{-3} s^{-1}), and that with dibucaine (18×10^{-3} s^{-1}).

The light intensity dependence of the kinetics of fluorescence quenching was determined. The rate constant increased with increasing light intensity, reaching saturation at approximately 300 μmol m^{-2} s^{-1} (Figure 4). Below this saturation point, the rate constant was higher in the chloroplasts containing zeaxanthin than in the dark-adapted control. The calculated maximum capacity of qE, qNo, showed a light intensity dependence somewhat different from that of the rate constant. Saturation was observed again around 300 μmol m^{-2} s^{-1}, but the difference arising from violaxanthin de-epoxidation was much greater for qNo than for the rate constant. It is clear that the quantum yield of qE formation is significantly enhanced by the presence of zeaxanthin. The extent of ΔpH was also dependent upon light intensity. There was little difference in the extent of formation of ΔpH in the presence and absence of zeaxanthin, and in fact, there seems to be a diminution in the light-activated chloroplasts. These data indicate that the light intensity dependence of the rate of fluorescence quenching is due to its effect of ΔpH.

A plot of the second-order rate constant versus ΔpH again shows the shift in the ΔpH dependence in the presence of

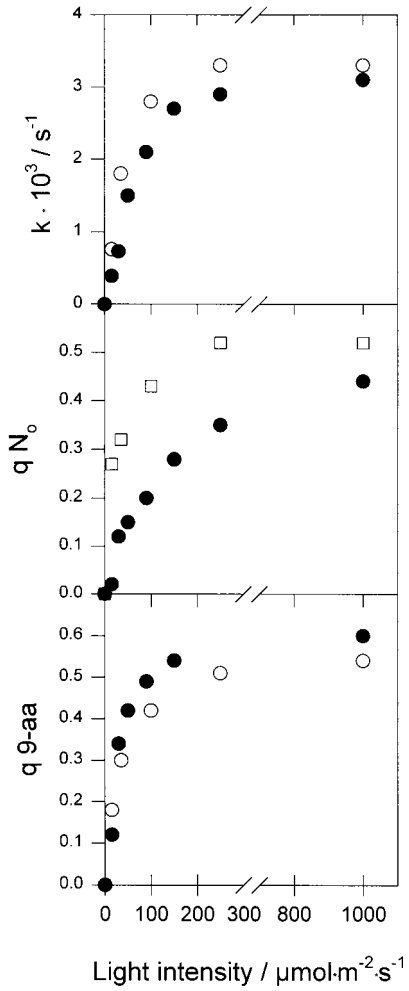

FIGURE 4: Dependencies of the calculated fluorescence quenching rate constant *k*, the amplitude of the maximum quenchable fluorescence, qNo, and the 9-aminoacridine fluorescence quenching, q9-aa, upon the intensity of actinic light calculated according to eq 3. Chloroplasts from light-treated leaves containing zeaxanthin (○). Chloroplasts from dark-adapted leaves containing only violaxanthin (●).

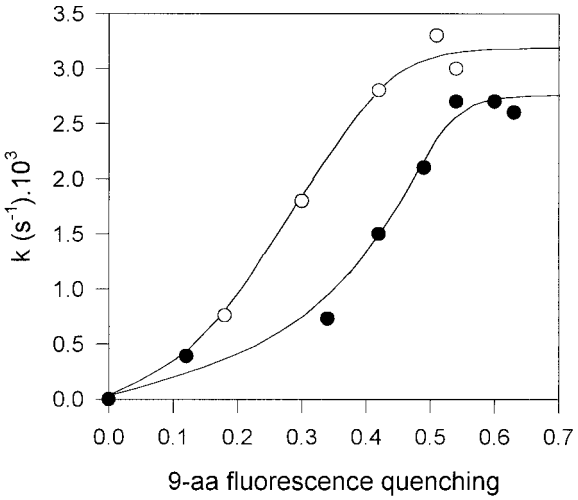

FIGURE 5: Relationship between the calculated chlorophyll fluorescence quenching rate constant *k* and the level of 9-aminoacridine fluorescence quenching. Chloroplasts containing zeaxanthin (○). Chloroplasts containing violaxanthin only (●).

zeaxanthin (Figure 5), similar to that shown in Figure 1 for the extent of qE. Thus, the conversion of violaxanthin to

zeaxanthin enhances the stimulatory effect of ΔpH on the rate of formation of qE. At saturating ΔpH, there is little difference in the rate constant with or without zeaxanthin.

DISCUSSION

The change in structure and function of the PSII antenna, as measured by the the level of formation of qE, has been shown to be described by the approaches classically applied to enzyme-catalyzed reactions. The process involves binding of two ligands (H^+ and zeaxanthin), and we have demonstrated that these ligands behave allosterically. Allostericity is evident from the influence of zeaxanthin upon the p*K* for qE formation. The sigmoidicity of the qE versus ΔpH titration curve indicates that proton binding shows positive cooperativity. We have shown that zeaxanthin acts as a positive effector, decreasing the concentration of H^+ required and at the same time reducing the degree of cooperativity. Similarly, antimycin A increases the ΔpH requirement for qE, and enhances cooperativity. Since there is previous evidence that qE involves a conformational change (*16*−*18*), it is suggested that cooperativity results from interactions between proteins in the PSII antenna. An oligomer of antenna subunits may provide multiple H^+ binding sites, protonation giving rise to the formation of the quencher via a conformational transition. It is suggested that cooperativity of H^+ binding is induced because of protein−protein interactions. The effect of zeaxanthin is to stabilize the protonated conformation, while antimycin stabilizes the unquenched unprotonated form.

While it has been demonstrated before that the level of qE is dependent upon both the ΔpH and zeaxanthin content, it is shown here conclusively that both these factors also control the rate of formation of quenching. In every case, the rate of quenching increases with an increase in irradiance, but reaches a ceiling level beyond which further increases in irradiance do not lead to increases in rate. This is not consistent with the suggestion that qE is a driven directly by light (*25*, *26*), although this does not exclude the possibility that another (minor under our conditions) component of quenching does arise from a direct photophysical effect on LHCII. Instead, the characteristics of light saturation are consistent with ΔpH being the principle driving force for qE formation. This driving force is modulated by other effectors. At a subsaturating ΔpH, antimycin A, a quenching inhibitor, slows the rate of qE formation, whereas the qE enhancers dibucaine and zeaxanthin increase the rate. It is important to point out that the rate of formation of quenching is therefore always linked to the amplitude of quenching. The mechanistic significance of this observation will be discussed in the following paper (*33*).

Previous data indicated that fluorescence quenching in leaves and chloroplasts fitted a single-component second-order reaction better than the sum of exponentials (*27*). The robustness of this kinetic model has been established by detailed examination of isolated chloroplasts in which the rate and amplitudes of quenching were varied by changes in irradiance, differences in de-epoxidation state, and the presence of exogenous modulators. In every case, the quenching of fluorescence followed second-order kinetics, the second-order rate constant depending on the particular condition. It is important to point out that the hyperbolic kinetics, and the linear reciprocal plot, indicate a binary reaction of the type $A + A \rightarrow 2A$. It has been observed previously that quenching of Chl fluorescence in isolated LHCII shares many features in common with qE. In the following paper, a detailed kinetic analysis of quenching in isolated LHCII is presented to enable the significance of the second-order reaction kinetics to be explored.

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