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Characterisation of the effects of Antimycin A upon high energy state quenching of chlorophyll fluorescence (qE) in spinach and pea chloroplasts

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Abstract. High energy state quenching of chlorophyll fluorescence (qE) is inhibited by low concentrations of the inhibitor antimycin A in intact and osmotically shocked chloroplasts isolated from spinach and pea plants. This inhibition is independent of any effect upon ΔpH (as measured by 9-aminoacridine fluorescence quenching). A dual control of qE formation, by ΔpH and the redox state of an unidentified chloroplast component, is implied. Results are discussed in terms of a role for qE in the dissipation of excess excitation energy within photosystem II.

Abbreviations: 9-AA_{max} = Maximum yield of 9-aminoacridine fluorescence; DCMU = 3(3,4-dichlorophenyl)-1,1-dimethylurea; F_{max} ± Maximum yield of chlorophyll fluorescence; hr = hour; PAR = Photosynthetically Active Radiation; Q_A = Primary stable electron acceptor within photosystem II; qE = High energy state quenching of chlorophyll fluorescence; qI = quenching of chlorophyll fluorescence related to photoinhibition; qP = Quenching of chlorophyll fluorescence by oxidised plastoquinone; qQ = photochemical quenching of chlorophyll fluorescence; qR = (F_{max}—maximum level of chlorophyll fluorescence induced by the addition of saturating DCMU); qT = Quenching of chlorophyll fluorescence attributable to state transitions.

Introduction

The yield of chlorophyll fluorescence eminating from the thylakoid membranes of higher plant chloroplasts has been shown to be reduced from a maximum level by five distinct processes (see [1, 9, 15] for reviews). These are; quenching resulting from oxidation of the primary quinone acceptor of photosystem II, Q_A (qQ), high energy state quenching (qE), quenching due to the oxidation of plastoquinone (qP), quenching induced by state transitions (qT) and quenching which occurs as a result of photoinhibition (qI). Since qQ is a reflection of the overall efficiency of charge separation within photosystem II, it is generally described as 'photochemical quenching' being distinct from the other four quenching processes which are essentially 'non-photochemical' in nature. The contribution of qQ towards the overall level of chlorophyll fluorescence quenching may be defined experimentally using the technique of light doubling [2, 21]. Under physiological conditions, the onset of photosynthesis gives rise to

both photochemical and non-photochemical quenching [2]. Although qE generally constitutes the major portion of non-photochemical quenching [8, 14, 21], other, less easily reversible, non-photochemical quenching mechanisms (qT and qI) become important under certain conditions, such as supra-optimal light intensities and low temperature [1, 21].

A relationship between quenching of chlorophyll fluorescence and the high energy state of the thylakoid membrane was first observed by Murata and Sugahara [19]. Whilst linearity between qE and a proton gradient across the thylakoid membrane has been demonstrated [3], the level of chlorophyll fluorescence quenching relative to ΔpH is diminished under certain circumstances. For example, osmotically shocked spinach chloroplasts frequently show minimal quenching, even with high ΔpH. Chlorophyll fluorescence quenching has also been shown to be more sensitive to the inhibitor antimycin A than is ΔpH [17]. These observations would seem to indicate that the relationship between qE and ΔpH is not obligatory and that what we presently refer to as high energy state quenching may in fact be partially, or even wholly, controlled by some other mechanism. In this study, an analysis has been made of the effects of antimycin A, upon the relationship between qE and ΔpH in intact and osmotically shocked chloroplasts from leaves of spinach plants (cv. Virtuosa) and pea plants (cv. Kelvedon Wonder).

Materals and methods

Intact spinach chloroplasts were prepared from the leaves of 18–21-day-old, hydroponically grown spinach plants (8-hr daylength, 200 μmoles quanta $m^{-2}s^{-1}$ of PAR), according to the method of Walker [22]. Chloroplasts were resuspended in a solution containing 0.33M sorbitol, 2 mM E.D.T.A., 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 50 mM Hepes buffer, brought to pH 7.6 using KOH.

Intact pea chloroplasts were isolated from the leaves of 11–14-day-old pea plants, grown in vermiculite under fluorescent light tubes (incident PAR of 48 μmoles quanta $m^{-2}s^{-1}$, 14-hr day length), according to the method of Cerovic and Plesnicar [4].

The addition of reagents during the course of assays was made using a microsyringe through the top of the reaction chamber. The volume of alcohol (ethanol) present in the reaction chamber was always less than 1%.

Intactness was greater than 80% when measured by the ferricyanide method. Broken chloroplasts were made by osmotically shocking intact chloroplasts in 5 mM $MgCl_2$ for 45 seconds before the addition of an equal volume of double strength resuspension medium. A chlorophyll concentration of 20 μg/ml was used for both intact and broken chloroplast assays.

Simultaneous measurements of oxygen uptake or evolution, 9-aminoacridine fluorescence and chlorophyll fluorescence were made using an apparatus similar to that described by Horton [8] with the following filter changes: The Actinic light source (580 μmoles quanta $m^{-2}s^{-1}$ of PAR) was filtered by a 660 nm

shortpass (Ealing 35-5446) and a Schott RG-610 glass filter. 9-aminoacridine fluorescence was excited through a Corning 7-39 glass filter and a 420 nm short pass (Ealing 35-5206) and defined by a 480 nm short pass (Ealing 35-5263), 450 nm broad band (Ealing 35-5024) and 457.5 narrow band (Ealing 35-3383) interference filters and two Corning 4-96 glass filters. With these filter combinations, filter overlap for the measurement of 9-aminoacridine was less than 10% at a concentration of 3 μM. Filter overlap for the measurement of chlorophyll fluoresence was negligible.

qE was defined, as a percentage of F_{max}, from the slow phase of the rise in chlorophyll fluorescence induced by the addition of 50 μM DCMU. qQ was measured using the 'light doubling' technique of Quick and Horton [22]. Saturating light pulses of 1.2 seconds duration and an intensity of 840 μmoles quanta $m^{-2} \cdot s^{-1}$ of PAR were delivered from a red DC light, defined using the same filter combination as the actinic light source, with a frequency of 7 seconds. The redox state of Q at any point was defined as $(1-(A/B)) \times 100$ where:

A = the total fluorescence yield at a point immediately prior to the light doubling spike and,
B = the yield at the top of the light doubling spike.

This gives a figure of 0 when Q is totally reduced tending towards 80 (% of F_{max}) when Q is totally oxidised.

All signals were fed through an Xcalibur XAD3 12 bit analogue to digital converter to a BBC model B microcomputer. Data was presented graphically on a Phillips monitor and then 'screendumped' using an Epson FX 80 printer.

Results

Figure 1A shows an induction curve of chlorophyll fluorescence in intact pea chloroplasts illuminated in the presence of saturating (0.1 mM) methyl viologen. This curve exhibits a characteristic slow quenching of fluorescence. The addition of 1.5 μM antimycin A prior to illumination (1B), greatly reduced both the rate and the degree of quenching. In 1C, antimycin A was added approximately thirty seconds into the induction period. The effect of this addition was to greatly reduce the rate of quenching. Calculation of qQ through light doubling suggested that the redox state of Q_A was unaffected by antimycin A, although a slight reduction in the rate of oxygen uptake (not shown) was observed. ΔpH, as measured by 9-aminoacridine fluorescence quenching, was unaffected by antimycin A under these conditions. These results indicate that antimycin A inhibits the formation of at least one non-photochemical quenching process without affecting the transthylakoid ΔpH.

The addition of 50 μM DCMU after two minutes illumination in Fig. 1 induced a rise in the level of chlorophyll fluorescence. The initial fast phase of this rise reflects the closure of 'Q traps', an effect similar to that of light doubling. The slow phase of the DCMU induced rise in fluorescence is generally

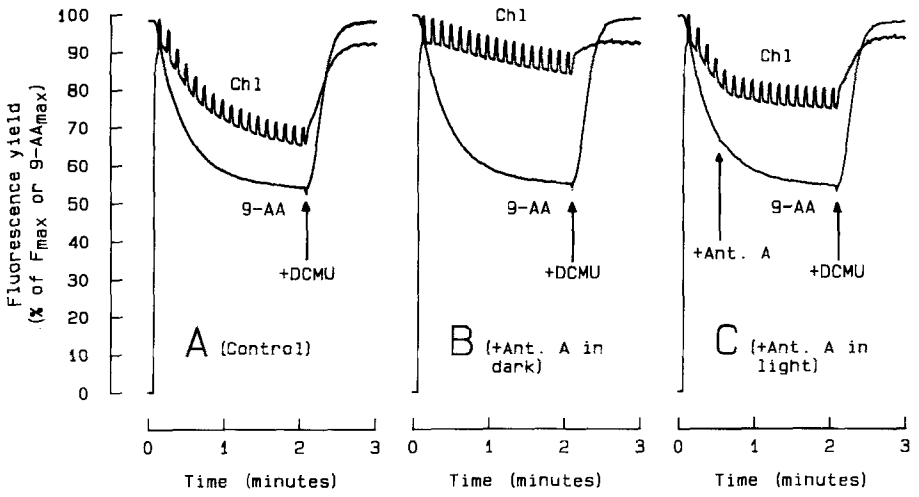

Fig. 1. Induction curves showing chlorophyll fluorescence (Chl) and 9-aminoacridine fluorescence (9-AA) in intact pea chloroplasts illuminated in the presence of 0.1 mM methyl viologen. A = No antimycin A. B = 1.5 μM antimycin A added prior to illumination. C = 1.5 μM antimycin A added after 30 s illumination. 50 μM DCMU added to A, B and C after 2 minutes in the light. The 'spikes' seen in the chlorophyll fluorescence trace are induced by a pulse of light of sufficient intensity to photo-reduce all molecules of Q_A and hence to remove qQ as a component of total quenching.

attributed to the reversal of qE. In Fig. 1A this slow phase is large, being equivalent to approximately 25% of the yield of chlorophyll fluorescence at F_{max}. The addition of antimycin A in the dark (Fig. 1B) reduces this figure to around 5% of the yield at F_{max}. In Fig. 1C, where antimycin A was added after 30 seconds illumination, a value intermediate between that from Fig. 1A and Fig. 1B was obtained (around 15%).

Titration of antimycin A concentration against qE produced an I_{50} of around 400 nM in intact pea chloroplasts (Fig. 2), 100 nM in intact spinach chloroplasts and 75 nM in broken spinach chloroplasts. These differences may be the result of variations in non-specific binding of antimycin A, e.g. to the chloroplast envelope or to differences in target site concentration. For comparison, an I_{50} of 50 nM would be roughly equivalent to one molecule of the inhibitor per photosystem II at the chlorophyll concentration used (20 μg/ml) assuming a molecular mass of 1 kDa for chlorophyll and 300 to 400 molecules of chlorophyll per photosystem II.

In Fig. 2, the difference between the height of F_{max} and the highest point of the DCMU-induced rise in chlorophyll fluorescence (qR), and 9-aminoacridine fluorescence are also plotted against antimycin A concentration, along with qE. Since the addition of saturating (50 μM) DCMU leads to the complete removal of qQ and qE, qR represents the difference in (qP + qT + qI) between F_{max} and the highest point of the DCMU induction curve. Plastoquinone is rapidly, and totally, oxidised in the presence of saturating DCMU and methyl viologen and therefore any variation in qR between samples can be attributed to differences in the levels of qT and/or qI.

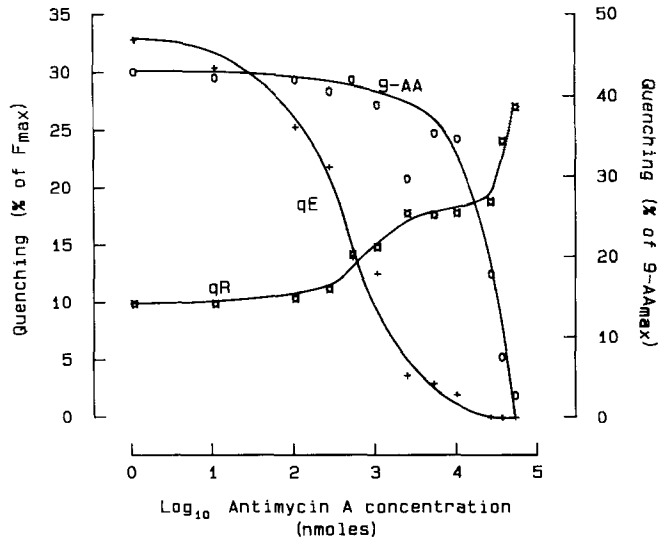

Fig. 2. Titration of antimycin A concentration against qE (+), qR (○) and 9-aminoacridine fluorescence (0) in inact pea chloroplasts illuminated in the presence of 0.1 mM methyl viologen. (see methods for details).

The relationship between qR and antimycin A concentration in Fig. 2 shows three distinct phases. Between zero and 5 μM antimycin A, an increase in qR is (qualitatively) well correlated with a decrease in qE seen over this region. Between 5 μM and 25 μM antimycin A, qR remains fairly constant. At antimycin A concentrations over 25 μM, an increase in qR is concurrent with a decrease in ΔpH. At these very high inhibitor concentrations, a reduction in the level of qQ with time is also seen (qQ remained at a constant level with time at antimycin A concentrations below 10 μM). These features are illustrated in Fig. 3. Also, whilst 85% to 90% of F_{max} could be recovered after five minutes relaxation in chloroplasts illuminated for ten minutes at antimycin A concentrations of 5 μM and less, at 10 μM and above variable fluorescence continued to decline in the dark.

Discussion

It is clear, from the results presented here, that antimycin A is a potent inhibitor of qE formation in isolated pea and spinach chloroplasts. The main point to be made from these results is that the relationship generally seen between ΔpH and qE is not obligatory since a high ΔpH (defined by 9-aminoacridine) can be maintained, without the formation of qE.

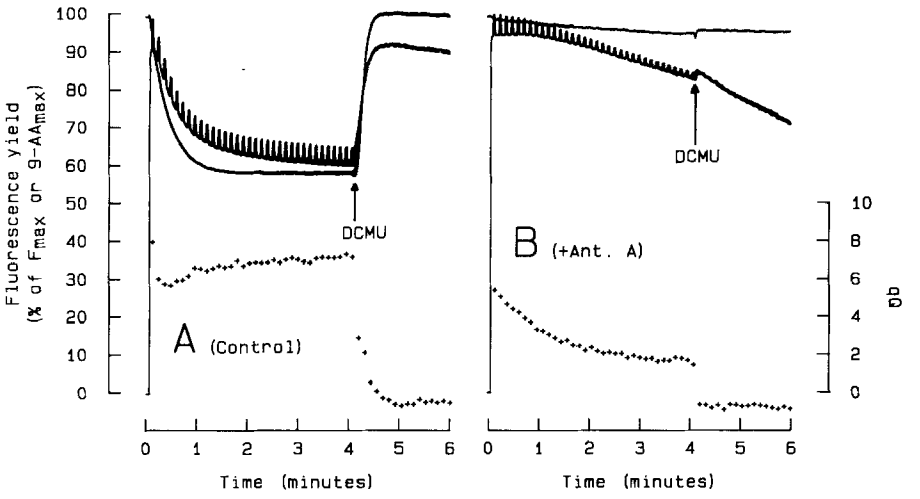

Fig. 3. Effect of high antimycin A concentration upon 9-aminoacridine fluorescence, chlorophyll fluorescence and qQ (+) in intact pea chloroplasts. A = No antimycin A, B = 50 μM antimycin A. DCMU was added at 4 minutes.

Antimycin A is also well documented as an inhibitor of redox reactions, the implication being that qE formation is under redox control. This control would probably operate alongside ΔpH (rather than as an alternative to ΔpH control) since:

a) there is little evidence within the literature to suggest that qE may be formed in the absence of ΔpH (one possible exception being a quenching of chlorophyll fluorescence induced by artificial acidification of the medium in the presence of an uncoupler [3]) and,
b) the addition of antimycin A does not reverse qE which has already formed (Fig. 1C) unless ΔpH is also reversed.

Redox state and ΔpH must operate together synergistically rather than additively since qE formation can be completely blocked by antimycin A, independently of the size of ΔpH.

The scheme below outlines the most widely accepted mechanism to explain the apparent relationship between ΔpH and qE formation.

1) The formation of a transthylakoid ΔpH leads to an efflux of cations from the thylakoid lumen in response to the increase in proton concentration.
2) Proton/cation (mainly Mg^{2+}) exchange occurs on the inner surface of the thylakoid membrane [16].
3) Protonation at the inner surface of the thylakoid membrane induces structural changes within photosystem II [14].
4) The yield of chlorophyll fluorescence decreases as a result of an increase in thermal de-excitation within photosystem II [15].

One possible explanation of the antimycin A effect upon qE formation is that the structural changes believed to occur within photosystem II are sensitive to the redox state of some component of the electron transport chain. Since antimycin A has little or no apparent effect upon the rate of linear electron flow, redox control of qE probably arises as the result of changes in the redox state of a component which is not involved in this pathway. On this basis cytochrome b_{563}, situated within the *b/f* complex, must be regarded as a possible target site since it only operates as an electron carrier in cyclic electron flow around photosystem I [20] and/or in the operation of a protonmotive Q-cycle within the *b/f* complex.

Antimycin A has also been shown to affect electron transfer reactions associated with cytochrome b_{559} [5] although at much higher concentrations than were used here. A number of correlations between pH, the level of variable chlorophyll fluorescence and the redox potential of this cytochrome have also been reported [10, 11]. From these observations, cytochrome b_{559} must also be seen as a possible redox state-dependent mediator between ΔpH and qE.

Although antimycin A is known to cause reduction of *b*-type cytochromes [7], Moss and Bendall [18] have found that, at low concentrations, the only target site is a 'ferredoxin quinone reductase' (FQR) which does not appear to be directly associated with either the *b/f* complex or photosystem II. In their experiments, ferredoxin mediated cyclic electron flow was reduced by 50% by an inhibitor concentration of approximately 100 nM. Since this figure is remarkably consistent with that seen here for the inhibition of qE formation, FQR must be regarded as the most likely primary site for the inhibition of qE formation by antimycin A.

Whilst qE is known to make a large quantitative contribution to the overall level of fluorescence quenching under many circumstances, it is unclear as to whether or not this quenching reflects a significant reduction in the amount of excitation energy being delivered to photosystem II reaction centres. The evidence presented here, of a link between redox state and qE formation, supports the concept of qE being involved in the control of electron transport in this way.

Krause and Behrend [13] have recently produced evidence to suggest that reducing the level of qE, using an uncoupler at high light intensity, leads to an increase in the level of qI. The correlation between the decrease in qE and increase in qR, seen in Fig. 2, at antimycin A concentrations below 5 μM is also most easily explained in terms of an increase in qI.

An alternative explanation, for the correlation between the decrease in qE and increase in qR seen over this range of antimycin A concentrations, is that it represents an increase in qT through a stimulation of protein phosphorylation, perhaps as a way of compensating for the reduction in qE (both a stimulation of protein phosphorylation by low antimycin A concentrations [6] and protection against photoinhibition by protein phosphorylation [12] have been reported).

An increase in qR at antimycin A concentrations above 25 μM (Fig. 2) occurs over a range where no qE quenching is evident and where quenching of 9-aminoacridine fluorescence is decreasing. Also, a progressive decrease in qQ over the period of illumination, which is not seen at low concentrations of antimycin A (Fig. 3A) is evident at concentrations above 25 μM (Fig. 3B). This latter feature strongly suggests that rapid photoinhibition, or photodestruction, is occurring over this range of concentration.

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