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The mechanisms of changes in Photosystem II efficiency in spinach thylakoids

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The parameter Φ_p (= rate of photosynthetic O_2 evolution/(incident light × qQ) reflects the efficiency of O_2 evolution by PS II reaction centres with respect to incident light, independent of the redox state of the primary electron acceptor Q_A (Weis, E. and Berry, J.A. (1987) Biochim. Biophys. Acta 894, 198–208). During whole chain electron transfer to methyl viologen in spinach thylakoids decreases in Φ_p of more than 50% were observed in the presence of a transthylakoid pH gradient. Evidence was obtained that there are two components of Φ_p decrease; one is associated with non-photochemical dissipation of chlorophyll excitation, observed as nonphotochemical fluorescence quenching (qNP), while a second component is not associated with fluorescence changes. The evidence for the latter was obtained using thylakoids that were suspended in low magnesium, or in the presence of antimycin A, so that they would develop only low levels of qNP. It is proposed that this component is due to electron cycling around the PS II reaction centre, and that the cycle is closely related to the transthylakoid pH gradient. The cycle can be observed during electron flow through PS II only, to *o*-phenylene diamine, and therefore does not appear to be controlled by the redox state of components beyond PS II in the electron transfer chain. Reversible Φ_p decreases observed in uncoupled thylakoids at low pH suggest that the cycle may be controlled by the pH of the intrathylakoid space, and not the pH gradient per se. Measurements made during the approach to steady state, in which the development qNP is slower that the formation of the pH gradient, are consistent with the suggestion that the contribution to Φ_p changes by qNP can be determined by the ratio of variable to maximum fluorescence yield (Genty, B., Briantais, J.-M. and Baker, N.R. (1989) Biochim. Biophys. Acta 990, 87–92).

Introduction

The overall efficiency of photosynthesis decreases with increasing light intensity. More specifically, the efficiency of O_2 evolution by Photosystem II (PS II) is reduced. This is reflected by a decrease in the parameter Φ_s = rate of photosynthetic O_2 evolution/absorbed light. A PS II centre in which the primary electron acceptor, Q_A, is reduced is unable to carry out charge separation, and is therefore said to be closed. At higher rates of electron transfer Q_A becomes progressively more reduced as components beyond PS II become rate limiting. Recent results, however, indicate that Q_A does not become as reduced as would be predicted if limitations occurred only beyond PS II [1]. It therefore appears that some limitation develops at the PS II centre itself.

At room temperature, chlorophyll fluorescence emmanates almost exclusively from chlorophylls associated with PS II, and is therefore a useful probe of PS II function. The fluorescence yield is quenched when chlorophyll excitation is utilised by photochemistry or dissipated by non-photochemical processes. Techniques have now been developed to resolve these processes, which are described by photochemical (qQ) and non-photochemical (qNP) quenching coefficients [2–4]. qQ indicates the proportion of excitation energy delivered to PS II centres which is captured by open centres. This

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Abbreviations: 9-aa, 9-aminoacridine; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Mes, 2-(*N*-morpholino) prophanesulfonate; PS I, Photosystem I; PS II, Photosystem II; Q_A and Q_B, primary and secondary stable electron acceptors of photosystem 2; qQ, photochemical fluorescence quenching coefficient; qNP, non-photochemical fluorescence quenching coefficient; qE, high-energy-state fluorescence quenching coefficient, q9aa, proportion of 9-aminoacridine fluorescence quenched; Φ_s, rate of photosynthetic O_2 evolution/absorbed light; Φ_p, Φ_s/qQ; $(F_v/F_m)_{ss}$, steady-state ratio of variable to maximum fluorescence yield.

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parameter therefore enables us to calculate the expected effect on Φ_s of PS II closure. If PS II efficiency were controlled by Q_A redox state only, then a linear relationship would be predicted between Φ_s and qQ [1,5]. Any deviation from linearity would indicate a change either in the proportion of absorbed light delivered to PS II centres, or in the functioning of the reaction centre itself. On this principle, Weis and Berry [1] defined the parameter $\Phi_p = \Phi_s/q\text{Q}$ which is a measure of PS II quantum yield independent of Q_A redox state. In other words a decrease in Φ_p indicates a decrease in efficiency in addition to the effect of Q_A redox state.

There have now been several reports that in vivo a decrease in Φ_p occurs which correlates with an increase in the extent of non-photochemical fluorescence quenching [1,6–10]. Two distinct interpretations of the data have emerged, both of which assume that the non-photochemical fluorescence quenching and the decrease in Φ_p are due to a common mechanism. The hypotheses depend on the relationships perceived between Φ_p and qNP.

Firstly, an inverse linear relationship has been observed between Φ_p and qNP [1,10]. By consideration of Butler's bipartite model of PS II [11,12,13], Weis and Berry [1] postulated that this was consistent with the existence of two distinct states of PS II. Under high light conditions, PS II centres would be converted to a second state, less efficient at O_2 evolution, which would also act as a quencher of chlorophyll excitation. Maximum qNP and minimum Φ_p would occur when all centres were in this second state.

Theoretical considerations predict that the steady-state ratio of variable to maximum fluorescence yield, $(F_v/F_m)_{ss}$, varies as the proportion of energy absorbed by the PS II light-harvesting chlorophylls that is delivered to the reaction centres. Genty et al. [6] observed a linear relationship between $(F_v/F_m)_{ss} \times q\text{Q}$ and Φ_s, which is equivalent to a linear relationship between $(F_v/F_m)_{ss}$ and Φ_p. They therefore suggested that PS II reaction centre function is controlled only by the redox state of Q_A and the efficiency of excitation delivery to open reaction centres.

In this paper we describe work carried out to determine the mechanism of Φ_p changes by studying in vitro systems. Although we observe changes in Φ_p directly related to qNP we also confirm the observation of Oxborough and Horton [14] that Φ_p changes can be observed independently of qNP. We analyse the data on the basis of both the theories outlined above, and discuss the implications with respect to the mechanism of qNP. We consider the control of Φ_p changes in vitro and how this may relate to PS II control in vivo.

Materials and Methods

Chloroplasts were isolated essentially as described in Ref. 15 from spinach grown hydroponically at 20 °C with a 12 h light period. 80 g of deribbed leaves were ground in 400 ml of 330 mM sorbitol, 5 mM $MgCl_2$, 2 mM sodium D-isoascorbate, 10 mM $Na_4P_2O_7$ (pH 6.5). The chloroplast pellet obtained after filtration and centrifugation was washed in 330 mM sorbitol, 10 mM Mes (pH 6.5) and resuspended in RS2 medium (330 mM sorbitol, 1 mM EDTA, 1 mM $MgCl_2$, 50 mM Hepes (pH 7.6)) at 1–2 mg chlorophyll · ml^{-1}. To ensure a high degree of intactness, the chloroplasts were layered over 10 ml RS2 medium containing 50% dialysed Percoll and pelleted by centrifugation at $3000 \times g$ for 5 min. The resulting chloroplasts, resuspended in RS2 at 1–2 mg chlorophyll · ml^{-1}, were greater than 80% intact by the method of Lilley et al. [16]. Chloroplasts were broken in the reaction chamber immediately before each measurement by a 15 s incubation in 0.5 mM EDTA, 10 mM Hepes (pH 7.6) and $MgCl_2$ as stated, after which sorbitol and Hepes (pH 7.6) were added to 330 mM and 55 mM respectively. Experiments were carried out at 20 °C with chloroplasts at 50 μg chlorophyll · ml^{-1} unless otherwise stated.

Simultaneous measurements of O_2 evolution/consumption and room-temperature chlorophyll fluorescence yield were performed essentially as described previously [2,17] using a water-jacketed 1 ml aluminium chamber with six ports (Hansatech DW2). Red actinic light was provided by a heat-filtered 250 W tungsten-halogen lamp (Volpi) with variable power supply, and defined by a 610 nm longpass (Corning RG610) and a 680 nm shortpass filter (Ealing Optics 35-5461). Chlorophyll fluorescence was excited using a measuring beam consisting of an array of yellow modulated LEDs (1 $\mu E \cdot m^{-2} \cdot s^{-1}$) and detected with a photodiode through two 715 nm longpass filters (Corning RG715) using a Hansatech modulated fluorometer [18]. A solid Perspex light guide through the water jacket was added to improve sensitivity of fluorescence detection. Signals were amplified and fed to a BBC microcomputer via a multi-channel analogue to digital converter.

In order to resolve photochemical and non-photochemical fluorescence quenching PS II centres were closed rapidly either by saturating pulses of light [2,3], or by the addition of 50 μM DCMU [4]. Saturating pulses of white light (duration 1.8 s, intensity 12000–24000 $\mu E \cdot m^{-2} \cdot s^{-1}$) were provided by one or two lamps identical to the actinic lamp, defined by a 680 nm short pass filter, and controlled by electronic shutters (Uniblitz). For each set of conditions the pulse intensity used was shown to be saturating under steady-state conditions with both high- and low-intensity actinic light, by the fact that a decrease in the intensity produced indistinguishable results. Similarly, the intensity of the measuring beam was shown to be sufficiently low to allow measurement of F_o fluorescence by the fact that an increase in its intensity had no observable effect on the measured ratio of variable to maximum fluorescence. The rapid fluorescence rise after the addition of DCMU was also used to calculate photochemical quenching. This was corrected for the extent of quenching by oxidised plastoquinone [19], which was estimated under the conditions of each experiment by comparison of fluorescence yield during a saturating pulse with that after DCMU addition in dark-adapted chloroplasts in the absence of nigericin.

0.4 μM 9-aminoacridine (9-aa) was included to allow estimation of the transthylakoid pH gradient [20]. 9-aa fluorescence was excited using a 50 W tungsten source, modulated by a Rofin chopper, and defined by a 420 nm shortpass filter (Ealing Optics 35-5206), with a Corning 5-58 and a Corning 7-39 filter. The signal was defined using a 480 nm shortpass filter (Ealing Optics 35-5263), a 460 nm narrow-band filter (Ealing Optics 35-3409), a 450 nm broad-band filter (Ealing Optics 35-5024) and two Corning 4-96 filters, detected by a photomultiplier tube (S11 response) and fed to a precision lock-in amplifier (EG & G, Brookdeal 9503) after preamplification. The quenching of 9-aa fluorescence, q9-aa, is expressed as a proportion of total initial fluorescence yield.

Fluorescence quenching coefficients were calculated essentially as defined in Ref. 21. F_o quenching, q_0, was calculated as $(F_o - F_o')/F_o$, where F_o is the dark level fluorescence at the start of the experiments, and F_o' is the minimum fluorescence level attained on turning off the actinic light. Photochemical quenching, qQ, was calculated as $(F_v)_s' - F_v')/(F_v)_s'$, where F_v' is the steady-state variable fluorescence and $(F_v)_s'$ is the maximum variable fluorescence level measured either using a saturating pulse of light, or at the end of the fast phase of relaxation of fluorescence after the addition of DCMU (see above). Non-photochemical quenching, qNP, was calculated as $((F_v)_m - (F_v)_s'/(F_v)_m$, where $(F_v)_m$ is the maximum variable fluorescence level attained during a saturating pulse of light applied at the start of the experiment on a dark adapted sample. F_v' and $(F_v)_s'$ were both calculated taking any quenching of F_o into account, i.e. using F_o'. (qNP defined here does not correspond to the qE defined in Ref. 21, as it incorporates quenching of variable fluorescence that arises by the mechanism responsible for q_o). The steady-state ratio of variable to maximum fluorescence, $(F_v/F_m)_{ss}$, was calculated as $(F_v)_s'/((F_v)_s' + F_o')$.

Photosynthetic yield, Φ_s, was calculated as the rate of electron transfer divided by the incident light intensity. PS II intrinsic yield, Φ_p was calculated as Φ_s/qQ.

Results

The control of PS II was studied during whole chain electron transfer to methyl viologen in spinach thylakoids in the presence and absence of nigericin.

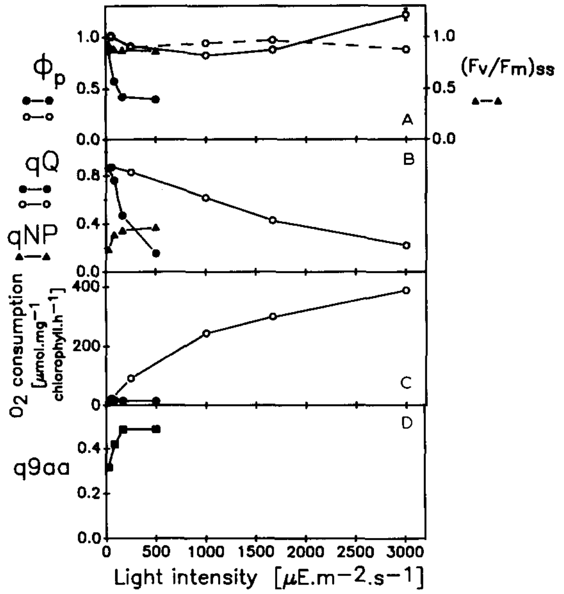

Fig. 1. Parameters measured during steady-state electron transfer to methyl viologen in the presence (open symbols) and absence (closed symbols) of 1 μM nigericin. Chloroplasts were broken in the presence of 2 mM $MgCl_2$ and resuspended in medium containing 100 μM methyl viologen and 100 μM NaN_3, at a concentration of 50 μg chlorophyll·ml^{-1} or, in the presence of nigericin, of 25 μg chlorophyll ·ml^{-1}. Steady state was attained after 5 min, or, in the presence of nigericin, after 20 s. qQ and qNP were resolved using 1 or 3 saturating pulses of light, or by the addition of DCMU. Results calculated by DCMU are shown only where they differ significantly (dashed lines). Φ_p and $(F_v/F_m)_{ss}$ are normalised with respect to the values at the lowest light intensity in the presence of nigericin. Results are the mean ± S.E. of three measurements.

Parameters measured over a range of light intensities are shown in Fig. 1. With methyl viologen as electron acceptor, electron transfer is measured as O_2 utilisation, thus artefacts due to O_2 consumption by the Mehler reaction are eliminated. In this, and later experiments, qQ and qNP were resolved both by saturating pulses of light, and, in separate experiments, by the resolution of slow and fast fluorescence relaxation on the addition of DCMU. When using DCMU, corrections were made for fluorescence quenching by oxidised plastoquinone (see Materials and Methods). The use of both methods allowed us to check for experimental artefacts. This was considered particularly important in the presence of nigericin, or when using phenylenediamine as electron acceptor, under which conditions it is more difficult to reduce Q_A with a saturating pulse. The results obtained using DCMU are shown only where they are significantly different from those obtained using saturating pulses. In these experiments quenching of 9-aa fluorescence was used as a measure of the transthylakoid pH gradient. The validity of 9-aa fluorescence quenching as a quantitative measure of the pH gradient is disputed [22]; hence, it is used here as a qualitative measure only.

As expected, in the presence and absence of nigericin, with increasing light intensity the rate of O_2 consumption increases and the extent of oxidised Q_A, indicated by qQ, decreases (Fig. 1b, c). In the absence of nigericin the rate of O_2 consumption is lower and reaches a maximum at about 100 $\mu E \cdot m^{-2} \cdot s^{-1}$, while qQ decreases much more rapidly than in the presence of nigericin. This is due to the formation of a transthylakoid pH gradient, observed as quenching of 9-aa fluorescence (Fig. 1d) which inhibits plastoquinone oxidation by the cytochrome *b-f* complex [23]. In this case non-photochemical fluorescence quenching (qNP) was observed (Fig. 1b), about half of which relaxed during 2 min dark adaptation (data not shown). As will be discussed later, under the conditions used in this experiment only low levels of qNP were apparent. In the presence of nigericin, the transthylakoid pH gradient was completely abolished, qNP was less than 10%, and had no reversible component.

In the absence of nigericin, the rate of O_2 consumption is lower than would be predicted from qQ. This is reflected by a decrease in Φ_p of more than 50% with increasing incident light which correlates both with the build-up of the transthylakoid pH gradient and the formation of qNP. In the presence of nigericin no significant decrease in Φ_p was observed, although the range of light intensities was extended to cover the same range of qQ values as in the absence of nigericin. A small rise in Φ_p was observed by the flash method, but not when DCMU was used. The rise is therefore assumed to be an artefact caused by an underestimation of qQ due to incomplete saturation by the pulse under these conditions. In this case errors would be significant at low qQ values only.

The relationships between Φ_p and both steady-state F_v/F_m ($(F_v/F_m)_{ss}$, and qNP were considered. In this system the decrease in $(F_v/F_m)_{ss}$ is small, and much less than the decrease in Φ_p. The decrease in Φ_p with respect to qNP (Fig. 2) is much greater than reported from in vivo studies, in which case Φ_p has never been observed to extrapolate to zero for qNP less than 1 [1,10]. In fact, the relationship shown here is not consistent with the theory put forward by Weis and Berry [1], as will be discussed later.

The presence of magnesium is necessary for maximum variable fluorescence yield and the development of qNP in thylakoids (Genty, B. and Briantais, J.-M., personal communication) [24,25]. Fig. 3 shows results obtained from chloroplasts broken in a range of magnesium concentration. Variable fluorescence yield, indicated by the dark adapted F_v/F_m was almost saturated at 2 mM magnesium (Fig. 3c), whereas qNP formation saturated at 30 mM magnesium (Fig. 3d). The increase in qNP was attributable to an increase in reversible quenching (dashed lines). Over the whole range of magnesium the extent of 9-aa quenching was fairly constant, showing only a slight decrease at the highest magnesium concentrations. For each magnesium concentration Φ_p was compared to the value measured in the presence of nigericin, which completely eliminated both qNP and

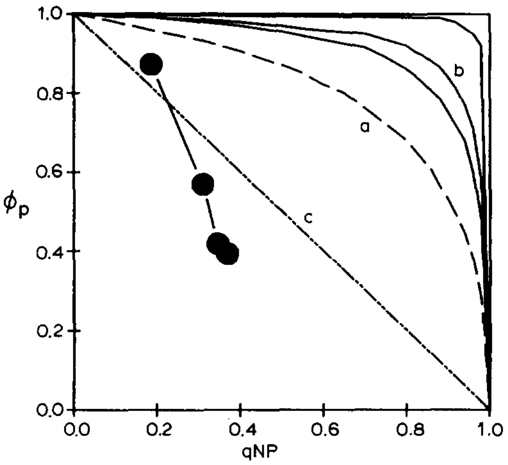

Fig. 2. The relationship between Φ_p and qNP during electron transfer to methyl viologen in the absence of nigericin. The data are those shown in Fig. 1. Predictions from the Butler model of energy transfer at PSII: (a) excitation dissipation in the light harvesting complexes and antenna. (b) excitation dissipation at the reaction centre (The three curves shown are representative of the possible relationships, but cover the complete possible range). (c) Two-state model for PS II. (See Discussion and Appendix for further details.)

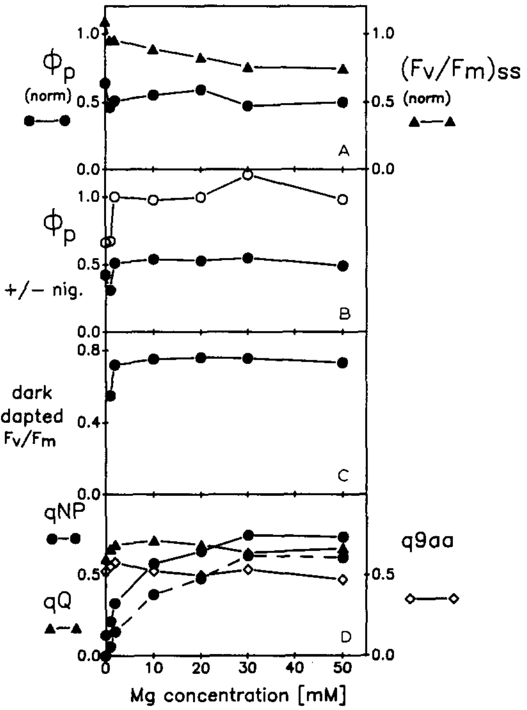

Fig. 3. Parameters measured during steady-state electron transfer to methyl viologen for chloroplasts broken in the presence of a range of $MgCl_2$ concentrations and exposed to 167 $\mu E \cdot m^{-2} \cdot s^{-1}$. Chloroplasts were suspended at a concentration of 50 μg chlorophyll $\cdot$ ml^{-1}. Steady state was attained after 5 min. qQ and qNP were resolved by a single saturating pulse of light. Similar results were obtained using DCMU. In Fig. 3a Φ_p and $(F_v/F_m)_{ss}$ are normalised with respect to the values measured in the presence of 1 μM nigericin.

quenching of 9-aa fluorescence. Over the whole range of magnesium concentration Φ_p decreased by about 50% with respect to that in the presence of nigericin (Fig. 3a). The values of Φ_p were independent of magnesium concentration above 2 mM magnesium, but an inhibition was observed at lower concentrations, both in the presence and absence of nigericin (Fig. 3b). This can be attributed to PS II damage under these conditions. Thus, despite a wide range of qNP values (0.1–0.7), the decrease in Φ_p with respect to that in the presence of nigericin was similar throughout (Fig. 3a). This demonstrates that decreases in Φ_p can be observed independently of qNP.

This experiment, together with that shown in Fig. 1 is consistent with a component of Φ_p change, independent of qNP, that is controlled by the transthylakoid pH gradient. It seems likely that the mechanism responsible for qNP also contributes to Φ_p changes, but it is not possible to determine to what extent from the data so far presented.

In spinach thylakoids the rate of qNP formation is much slower than the build up of the transthylakoid pH gradient. Parameters measured during the first minutes of exposure to light can therefore be used to distinguish between the effects on Φ_p of the pH gradient and qNP. Fig. 4 shows Φ_p, qNP and the pH gradient during the first few minutes of illumination for chloroplasts broken in low and in high concentrations of magnesium. In the low magnesium thylakoids qNP formation is not extensive (Fig. 4c). In this case, associated with the rapid formation of the transthylakoid pH gradient, Φ_p decreases rapidly, and only small changes are observed once the pH gradient has been established. This is consistent with most of the Φ_p decrease being controlled directly by the pH gradient. However, in the high-magnesium thylakoids in which qNP is high, there is a significant decrease in Φ_p after the transthylakoid pH gradient has reached steady state, suggesting that there is a significant effect of excitation dissipation (Fig. 4a). The measurements were repeated in the presence of 1 μM antimycin A, which inhibits qNP formation [14]. In this case, the slower changes in Φ_p were eliminated and Φ_p changes behaved in a similar manner to the pH gradient (Figs. 4b, d). Φ_p divided by $(F_v/F_m)_{ss}$ has also been plotted in each case in order to examine the possibility that the effect of qNP is related to $(F_v/F_m)_{ss}$ as suggested by Genty et al., in which case this would represent Φ_p corrected for the effect of qNP. With this correction it is noteworthy that the parameter correlates with the pH gradient at both magnesium concentrations, with or without antimycin A.

The data presented above suggest that in vitro there are two processes contributing to the decrease in Φ_p: non-photochemical excitation quenching, and a second process which is completely independent of non-photochemical excitation quenching. It appears that the contribution of non-photochemical excitation quenching can be determined, at least approximately, by considering the parameter $(F_v/F_m)_{ss}$. Further experiments were carried out to obtain more information about control of the second process.

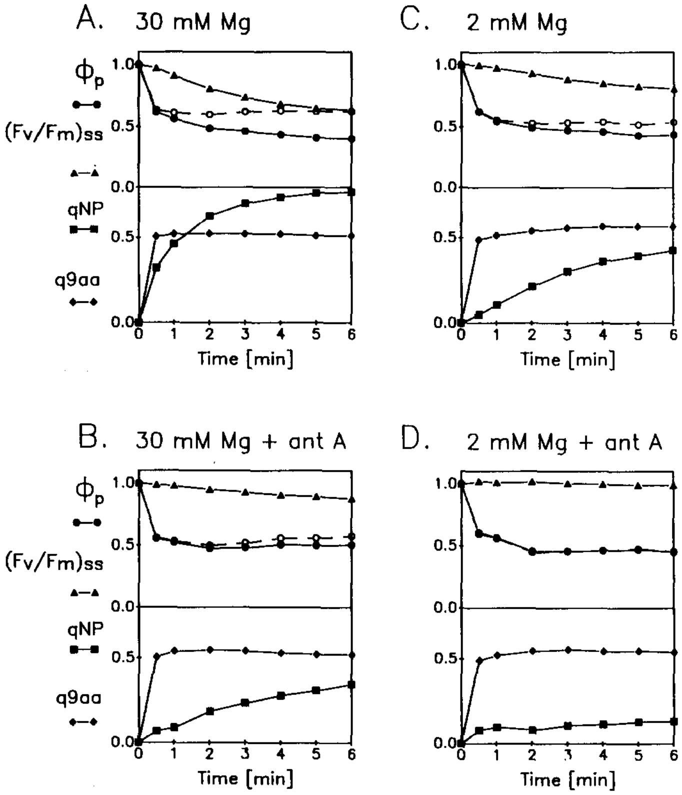

Fig. 4. The development of the transthylakoid pH gradient, Φ_p changes and qNP during the approach to steady state in the presence of 278 $\mu E \cdot m^{-2} \cdot s^{-1}$. Chloroplasts were broken in the presence of 2 mM (a, c) or 30 mM (b, d) $MgCl_2$, and were assayed in the absence (a, b) or presence (c, d) of 1 μM antimycin A. For the results shown qQ and qNP were resolved by saturating pulses of light, but similar results were obtained using DCMU. Separate samples were used to determine the extent of F_o quenching during the run, and this was taken into account for the calculation of qQ and qNP. Φ_p and $(F_v/F_m)_{ss}$ are normalised to the values measured in the presence of nigericin. Φ_p divided by $(F_v/F_m)_{ss}$ is also shown (open symbols, dashed lines).

Fig. 5 shows data obtained during electron flow to o-phenylene diamine, which accepts electrons from the plastoquinone pool. The experiments were carried out in the presence of 0.5 μM DBMIB to prevent any whole chain electron flow through to ferricyanide, which was included to oxidise the phenylene diamine. At this concentration no quenching of fluorescence by the DBMIB could be observed. Fluorescence parameters were measured both by saturating light pulses, and by DCMU addition, but no significant difference was observed in the results. As there is no electron flow through the cytochrome b-f complex, photosynthetic control is not observed, and indeed, as the activity of phenylene diamine is inhibited by uncouplers [26], qQ is lower in the uncoupled state. In the absence of nigericin Φ_p decreases in the presence of the transthylakoid pH gradient. This decrease is greater than the decrease in $(F_v/F_m)_{ss}$, suggesting that there is a component independent of qNP. These results are consistent with the conclusion from Fig. 1 that Φ_p changes are not controlled by the redox state of components beyond PS II in the electron transfer chain, but can be produced by changes in components prior to the cytochrome *b-f* complex.

Reversible decreases in Φ_p (again greater than the decrease in $(F_v/F_m)_{ss}$) can be observed in thylakoids uncoupled by the addition of both nigericin and valinomycin and suspended in low pH medium (Fig. 6). This suggests that Φ_p changes are controlled by the pH of the intrathylakoid space. In this experiment it was found that bright pulses of light resulted in irreversible quenching. It was assumed that inhibition of the water-splitting complex by low pH led to damage by oxidised P680 under these conditions. Consequently, qQ and qNP were resolved by DCMU addition only.

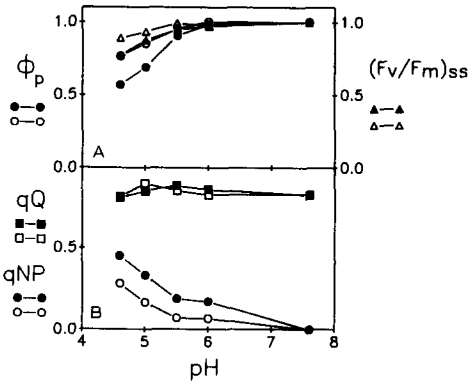

Fig. 6. The effect of pH on Φ_p in thylakoids. Chloroplasts were broken in the presence of 10 mM $MgCl_2$ and resuspended to 50 μg chlorophyll·ml^{-1} in medium containing 100 μM methyl viologen, 100 μM NaN_3, 1 μM nigericin and 1 μM valinomycin. Both the breaking and final resuspension medium were adjusted to the following pHs with the respective buffers: pH 4.6 (citrate), pH 5.0 (citrate), pH 5.5 (Mes), pH 6.0 (Mes) and pH 7.6 (Hepes). After a 2 min incubation the suspension was diluted with medium of appropriate pH to maintain the same pH (closed symbols) or to give a final pH of 7.6 (open symbols). The final chloroplast concentration was 27 μg chlorophyll·ml^{-1}. qQ and qNP were resolved using DCMU. The actinic light intensity was 167 μE·m^{-2}·s^{-1}. Φ_p and $(F_v/F_m)_{ss}$ were normalised with respect to the values measured in chloroplasts maintained at pH 7.6.

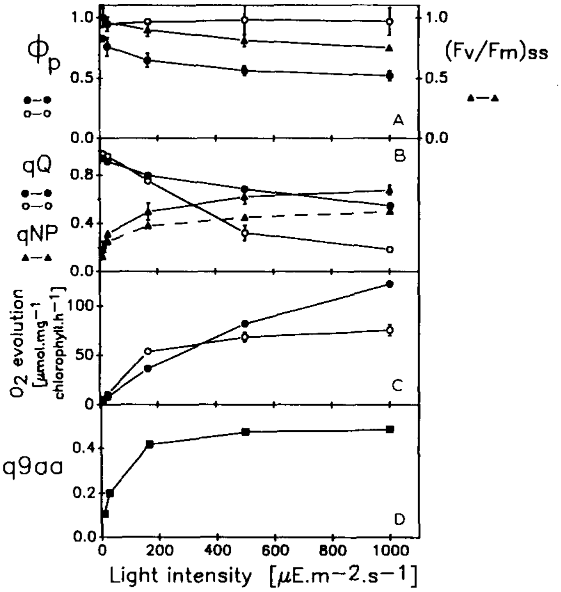

Fig. 5. Parameters measured during steady-state electron transfer to *o*-phenylene diamine in the presence (open symbols) and absence (closed symbols) of 1 μM nigericin. Chloroplasts were broken in the presence of 10 mM $MgCl_2$ and resuspended in medium containing 500 μM *o*-phenylene diamine, 3 mM $K_3Fe(CN)_6$ and 0.5 μM DBMIB at a concentration of 50 μg chlorophyll·ml^{-1}. Steady state was attained after 5 min, or, in the presence of nigericin, after 20 s. Other conditions are as for Fig. 1.

Discussion

During photosynthetic electron transfer in vitro, the build-up of the transthylakoid pH gradient clearly has two opposing effects on the redox state of Q_A; inhibition of the oxidation of plastoquinone by the cytochrome *b-f* complex causes Q_A to become more reduced [23], while a decrease in the efficiency of the PS II reaction centre, reflected in the parameter Φ_p, decreases the extent of Q_A reduction. In this paper we have considered the mechanism and control of Φ_p changes. Whereas previously it has been considered that Φ_p changes are brought about by a single mechanism, we have extended the earlier observations of Oxborough and Horton [14] to show that two mechanisms are apparent in vitro. The dissipation of excitation observed as qNP results in a Φ_p decrease, but there is a second mechanism that has no effect on fluorescence yield.

The evidence for the second mechanism comes from experiments carried out under conditions in which the qNP formation by thylakoids is low. One method used for manipulating the extent of qNP was by magnesium concentration during chloroplast breakage. As observed previously, the magnesium optimum for qNP was found to be greater than for maximum variable fluorescence yield [24]. The saturating concentration for qNP of 30 mM was higher than that observed by Briantais et al. although similar to the concentration quoted by jennings [25]. However the nature of the quenching observed by Jennings et al. is not clear. At the appropriate magnesium concentrations the thylakoid preparation used in this study was capable of extensive qNP formation, most of which was reversible (Fig. 3). The thylakoids used for Figs. 1 and 2 were suspended at low magnesium and therefore showed only low levels of qNP.

The effect of the quenching of excitation responsible for qNP on the activity of PS II depends on the mechanism of the quenching. The Butler model of energy transfer at PS II [11,12,13] provides a useful framework for interpreting data. The relationships between Φ_p and qNP predicted by the Butler model in the case of energy dissipation in the light harvesting complexes and antenna, or of energy dissipation at the reaction centre, are shown in Fig. 2 (lines (a) and (b), respectively). In both cases the effect on Φ_p is great only for large qNP. The two-state model suggested by Weis and Berry [1] would result in a straight line relationship intercepting the reaction centre curves at two places, such as line (c) in Fig. 2, or a line with smaller slope. This 2 state model is not consistent with the data shown in Fig. 2, and, in fact, as mentioned above, we assume that a second process is affecting Φ_p in this case. The data shown in Fig. 4 can give us more direct evidence for the specific relationship between Φ_p and qNP. Figs. 4b and c show that a change in qNP from 0 to 0.4 has little effect on Φ_p, whereas an increase from 0.4 to 0.8 has a much more significant effect (Fig. 4a). This is inconsistent with the 2 state model, but could be explained by either of the other 2 hypotheses.

In addition, the Butler model predicts that in the case of energy dissipation in the light-harvesting complexes and antenna, Φ_p would be proportional to $(F_v/F_m)_{ss}$ as suggested by Genty et al. [6]. Whereas, for reaction centre quenching Φ_p would decrease less than $(F_v/F_m)_{ss}$, and for the two state model Φ_p would decrease more than $(F_v/F_m)_{ss}$. The relationship between $\Phi_p/(F_v/F_m)_{ss}$ and the pH gradient shown in Fig. 4 is therefore consistent with qNP being due to energy dissipation in the light-harvesting complexes and antenna. This supports data presented elsewhere [27].

In the discussion so far, we have considered qNP as a single component. Measuring the reversal of quenching during 2 min dark adaptation gives a lower estimate for the contribution of high-energy-state quenching (qE) [4] (see Figs. 1,3, 5). In all cases measured, there was a component of irreversible or slowly reversing quenching, although this was a smaller proportion of total quenching when qNP was large (Fig. 3). It is therefore possible that qNP considered here has a contribution from photoinhibitory quenching. If qNP overestimates qE in Fig. 1, then this strengthens the argument that the mechanism responsible for qE is not sufficient to account for observed changes in Φ_p. State transitions are also relevant with respect to the use of the parameter $(F_v/F_m)_{ss}$, as theoretically a state transition could effect Φ_p with no change in $(F_v/F_m)_{ss}$. However, state transitions do not occur in vitro in the absence of ATP [28], as is the case in these experiments.

Any form of energy dissipation prior to charge separation at PS II would result in quenching of fluorescence. Thus, at present, the only possible mechanisms that we can envisage that could change Φ_p independently of fluorescence parameters in these experiments (using methyl viologen as electron acceptor) would be an electron cycle operating around PS II, or a back-reaction (as long as this does not result in luminescence). Electron cycling within PS II has been suggested before as a protective mechanism during high incident light [14,29–35]. The potential for protection is clear, as a cycle could prevent over-reduction of the acceptor side of PS II, and could also provide a rapid route for the re-reduction of P680. Recently, evidence has been presented that P680 is rapidly reduced by an adjacent chlorophyll [36]. In this case a cycle might act via such a chlorophyll. Evidence that the high-potential form of cytochrome *b*-559 can be both oxidised and reduced by PS II indicates that it may also be involved in such a mechanism. At this time there has been no direct demonstration that electron cycling around PS II can occur at a sufficient rate to account for the Φ_p changes observed here. (We will refer to this mechanism as a cycle in further discussion, although a back-reaction has not been ruled out.)

Although, both the cycle and photosynthetic control at the cytochrome *b-f* complex are related to the transthylakoid pH gradient, they are clearly independent. This was demonstrated by using phenylene diamine as electron acceptor, in which case photosynthetic control was not apparent, but the decrease in Φ_p was still too large to be accounted for by qNP alone. The results of Fig. 6 indicate that the cycle may be controlled by the pH of the intrathylakoid space, rather than the pH gradient per se.

The conditions under which the cycle has been demonstrated in this paper are in many ways non-physiological. In order to show Φ_p changes independently of qNP, it was necessary to decrease the extent of qNP formation by using low magnesium conditions, or by the addition of antimycin A. However, the fact that Φ_p changes in high magnesium, in the absence of antimycin A, were greater than predicted for qNP, strongly suggests that the cycle operates under these conditions as well. As there is no dissipation of the pH gradient by ATP synthesis we would expect the pH gradient to be greater than under most conditions in vivo. An important question to consider is, under what conditions, if any, the cycle would operate in vivo. In spinach leaves and in intact chloroplasts during CO_2 dependent O_2 evolution, we found that Φ_p measured over a range of light intensities was proportional to $(F_v/F_m)_{ss}$ (results not shown), such that the changes could be explained completely by excitation dissipation. This agrees with the observations of Genty et al. However, it is clear from some reports in the literature that the decreases in Φ_p observed are greater than the decreases in $(F_v/F_m)_{ss}$ [1,10], and we have obtained similar data for the green alga *Dunaliella* C9AA [37] and for protoplasts during induction [38]. This is consistent with the operation of a PS II cycle, although, unlike the results of this paper, it could also be explained by the Mehler reaction. By another method, evidence has been obtained recently of a cycle operating during high light in green algae [39].

Although both qNP and the cycle are closely related to the transthylakoid pH gradient, it is not yet clear why this should lead to the frequent observation of an inverse linear relationship between Φ_p and qNP [1,10]. This may become clearer after more detailed examination of the relationship between qNP and the pH gradient, and between the cycle and the pH gradient (e.g., Ref. 40).

Appendix

Theoretical calculations from the Butler model

The relationships between Φ_p and qNP predicted in the case of energy dissipation in the antenna or reaction centre (*a* and *b* in Fig. 2) were calculated using the Butler model [13]. In this model the following processes are considered – in the antenna and light-harvesting complexes: k_F, excitation dissipation by fluorescence; k_D, excitation dissipation as heat; k_T, excitation transfer to the reaction centre; and processes at the reaction centre; k_p, photochemistry; k_d, excitation dissipation as heat; k_t, excitation transfer to the antenna. k_p is set to zero in closed centres. Processes at the antenna, and at open and closed reaction centres are normalised to give terms of the form ψ_I, $\psi_{i(o)}$ and $\psi_{i(x)}$ respectively.

Fluorescence yield is given by:

$$\Psi_F = \frac{\psi_F[A(1-\psi_T\psi_{t(x)})+(1-A)(1-\psi_T\psi_{t(o)})]}{(1-\psi_T\psi_{t(o)})(1-\psi_T\psi_{t(x)})} \qquad (1)$$

where A is the proportion of total centres which are open. Maximum fluorescence yield, Ψ_{F_m}, and dark level fluorescence yield, Ψ_{F_o}, are calculated by setting A to 0 and 1, respectively.

Photochemical yield is given by:

$$\Psi_p = \frac{A\psi_T\psi_p(1-\psi_T\psi_{t(x)})}{(1-\psi_T\psi_{t(o)})(1-\psi_T\psi_{t(x)})} \qquad (2)$$

Thus Φ_p is calculated as $\Psi_p/q\text{Q}$, where $q\text{Q} = (\Psi_{F_m} - \Psi_F)/(\Psi_{F_m} - \Psi_{F_o})$. It can easily be shown that this is equivalent to calculating Φ_p as Ψ_p with A set to 1.

Initial ψ values were chosen to give $\Psi_{Fv}/\Psi_{Fm} = 0.8$ ($\Psi_{Fv} = \Psi_{Fm} - \Psi_{Fo}$), and $\Psi_{p(A=1)} = 0.95$ by methods set out in Ref. 13. The whole range of possible ψ values was considered. Parameters were then calculated for increasing values of k_D, for dissipation in the light-harvesting complexes and antenna, or k_d, for dissipation at the reaction centres. qNP was calculated as $(\Psi_{Fv(\text{init.})} - \Psi_{Fv})/\Psi_{Fv(\text{init.})}$, where $\Psi_{Fv(\text{init.})}$ was calculated using the initial ψ values.

It was found that in the case of dissipation at the antenna, the relationship between qNP and Φ_p was constant for any given Ψ_{Fv}/Ψ_{Fm}. In the case of dissipation at the reaction centre, the relationship depended on Ψ_{Fv}/Ψ_{Fm} and the degree of energy cycling between the antenna and reaction centre (determined by the value of ψ_p [13]). The curves shown in Fig. 2 cover the complete possible range.

Energy transfer between antenna of different PS II centres was considered, but has no effect on any of the theoretical relationships used in this paper.

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