The quenching of photosystem II fluorescence does not protect the D1 protein against light induced degradation in thylakoids

S. Santabarbara[a], R. Barbato[b], G. Zucchelli[a], F.M. Garlaschi[a], R.C. Jennings[a,*]

[a] *Centro C.N.R. Biologia Cellulare e Molecolare delle Piante, Dipartimento di Biologia, Università di Milano, Via Celoria 26, 20133 Milan, Italy*
[b] *Dipartimento di Scienze e Tecnologie Avanzate, Università del Piemonte Orientale, Corso Borsalino 54, 15100 Alessandria, Italy*

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Abstract In spinach thylakoids, the quenching of the singlet excited state in the photosystem II antenna by *m*-dinitrobenzene does not change the rate of the light induced degradation of the D1 reaction centre protein and offers only limited protection against photoinhibition itself. These results are discussed in terms of the role of non-photochemical quenching as a photoprotective strategy.

Key words: Photoinhibition; D1 degradation; Excited state population; Fluorescence quenching; Thylakoid

1. Introduction

Plants are often exposed, in their natural environment, to photon fluences which exceed the electron transport capability, due to slow reoxidation of the plastoquinone pool by the cytochrome (Cyt) b_6f complex [1]. These conditions promote oxidative damage mainly to photosystem II (PSII) which leads to the inactivation of photosynthetic electron transport and which is followed by the proteolytic degradation of the 32 kDa reaction centre protein D1 [2–4] mediated by the recently discovered proteases DegP2 [5] and Ftsh [6]. This latter process is thought to initiate the disassembly of damaged reaction centre complexes as part of a repair mechanism (see [7] for a review). The degradation of the other reaction centre protein, D2, as well as the formation of cross-linking products between D1 and D2, D1 and Cyt b_{559}, and D1 and CP43 [8,9], have been also reported in the literature [10,11]. Both the inactivation of the PSII mediated electron transport chain and the protein degradation can be induced by two different mechanisms involving the acceptor and the donor side of the photosystem (for reviews see [4,7]). When photoinhibition is determined by damage at the oxidising side of PSII, it is almost oxygen independent and probably related to the accumulation of highly oxidising species like P^+_{680}, Tyr^+_{161} and/or the accessory chlorophyll (Chl) cation (Chl^+_Z) [12]. In this case the D1 protein is specifically cleaved and two fragments, of apparent molecular weight 24 and 9 kDa, have been detected [13], corresponding respectively to the C- and N-terminus of the protein [14]. On the other hand, fragments of 23 [15] and 10 kDa [16] have been detected during acceptor side induced photoinhibition corresponding to the N- and C-terminus of the protein respectively. Other fragments around 16 kDa have also been detected, during both in vivo [17] and in vitro experiments [18], but their relation to the particular kind of photoinhibition is less clear. Degradation of D1 during acceptor side photoinhibition is dependent on the presence of oxygen [19], and is accompanied by singlet oxygen ($^1O_2$) production [20,21]. Therefore it was suggested that the D1 degradation be triggered by an intermediate, activated by the interaction with $^1O_2$ [4,7].

$^1O_2$ is generated in photosynthetic systems by the interaction of the excited triplet state of Chl and molecular oxygen (see [22] for a review). The triplet state in Chl–protein complexes is kept at very low levels by extremely efficient ($\sim$100%) quenching by carotenoids [23]. The population of a triplet state in the reaction centre of PSII originating from the charged separated state [$P^+_{680}Pheo^-$], the recombination triplet, has been demonstrated in conditions in which the primary quinone acceptor is double reduced and protonated [24]. As the P_{680} recombination triplet, when populated in the quinone depleted D_1D_2Cyt b_{559} complex [25], is efficiently quenched by oxygen at physiological temperatures, it has been proposed that it plays a key role in photoinactivation [25,26]. On the other hand, while the rate of the PSII reaction centre triplet population is expected to be proportional to the singlet excited state population in the PSII antenna bed, it was recently reported that when the excited state levels were lowered, either by added singlet quenchers [27–29] or spillover [27], in isolated thylakoids, only a very limited protective effect against the photoinhibition induced loss of PSII photochemical activity was detected [27–29]. These findings were interpreted in terms of a small population of Chl molecules, uncoupled, or poorly coupled to the PSII antenna bed, and therefore almost insensitive to the quenchers [27–29]. Moreover the photoinhibition action spectrum was shown to be 3–4 nm blue shifted compared to the bulk absorption of PSII [28]. The presence of uncoupled/weakly coupled Chl molecules is also indicated by both the steady state [28] and time resolved [30] quenching analysis of thylakoid fluorescence emission in which a small amplitude, blue shifted component with limited quencher sensitivity was demonstrated.

In view of the close relationship between photoinhibition and the subsequent D1 protein degradation we have investigated the sensitivity of this latter process to the antenna excited state population. It is demonstrated that when the excited state levels are lowered by a singlet quencher, D1 protein

*Corresponding author. Fax: (39)-2-2660 4399.
E-mail address: robert.jennings@unimi.it (R.C. Jennings).

Abbreviations: PSII, photosystem II; P_{680}, PSII reaction centre; $^1O_2$, singlet oxygen; Chl, chlorophyll; DNB, *m*-dinitrobenzene; F_M, maximal fluorescence yield; F_o, fluorescence yield at open reaction centres

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degradation, which is initiated long after photoinhibition, is unaffected.

2. Materials and methods

Thylakoids were prepared from freshly harvested spinach leaves as previously described [31] and Chl concentration determined with the extinction coefficients given by MacKinney [32]. *m*-Dinitrobenzene (DNB) was added from a concentrated alcohol solution with the final solvent concentration being less than 1% (w/v).

Photoinhibition treatments were performed in a 1 cm cuvette at a Chl concentration of 20 μg/ml at 4°C. The light source was a 900 W xenon arc lamp, filtered through 5 cm of water and a heat plus UV removing filter (Balzer, Calflex-C). The intensity of the incident radiation was 45 mW/cm^2. This high intensity was used in order to shorten the experimental treatment times. It was previously demonstrated that the light fluence/reciprocity for loss of PSII photochemical activity in this thylakoid system is valid at this intensity [27]. Fluorescence induction measurements were performed in a home built apparatus, as previously described, except that the excitation wavelength was 638 nm (Balzer B-40, FWHM 10 nm) and the emission was selected through a 700 nm interference filter (Balzer B-40, FWHM 10 nm) and a cut-on RG695 (Schott). Measurements of treated sample were performed after a dark adaptation time of 15 min at 4°C to allow complete Q_A reoxidation. To correct for the amount of Chl photooxidation which became detectable after 10–15 min of light treatment and attained values of up to 20% after 30 min, the maximal fluorescence yield (F_M) values are given as the ratio of the measured fluorescence intensity to the product of the integral of the sample absorption spectrum and the filter bandshape. The absorption spectra were measured in a home built spectrometer equipped with an EG&G OMAIII detector, as previously described [33]. In some control experiments fluorescence was detected in the same apparatus used for the photoinhibition treatment by placing a CS 4-96 (Corning) broad band filter in the light path, and detecting the fluorescence at 90° through an interference filter (λ_{max} 684 nm, Balzer B-40, FWHM 10 nm) with a highly sensitive photomultiplier (EMI-thorn B2/RFI). The light intensity was measured using a radiometer equipped with a bolometer as a sensor (YSI-Kettering Mod. 65).

For the immunoblot experiments thylakoids were solubilised, after 1 h of dark adaptation at room temperature (20°C), according to Laemmli [34], and the proteins were separated in a 6 M containing urea 14% (w/v) polyacrylamide gel with 0.4 μg Chl being loaded per well. After electrophoresis, proteins were transferred to a polyvinylidene fluoride membrane and probed with antibodies raised against the proteins D1 and Cyt *f*. Immunoreactions were detected by enhanced chemiluminescence (Pierce, Supersignal kit) with a photographic film (Kodak BioMax Light-1) and quantified by the analysis of several such films with a Bio-Rad GelDoc 2000 imaging densitometer.

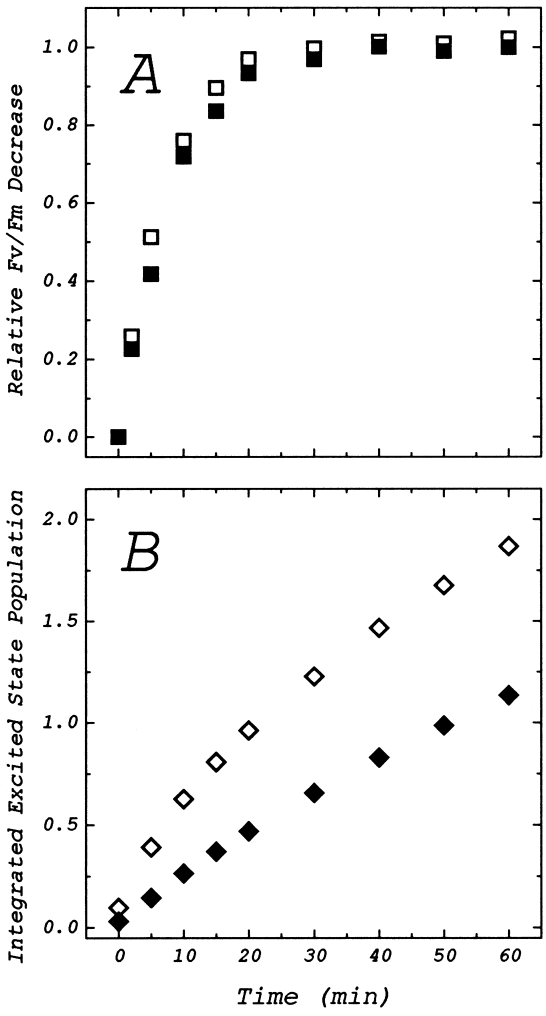

Fig. 1. A: The kinetics of the light induced loss of maximal photochemical yield (F_V/F_M). The values are normalised to the initial F_V/F_M values, which for the control were 0.75 and for DNB (500 μM) 0.43. B: The kinetics of the excited state population, as indicated by F_M, integrated over the experimental time. Open symbols are control samples, closed symbols are samples incubated with 500 μM DNB.

3. Results

Fig. 1 shows the kinetics of PSII photoinhibition, as monitored by the F_V/F_M ratio, for samples in the absence and presence of a singlet quencher which reduced the initial antenna excited state population by 67%. It was previously demonstrated that this fluorescence ratio is linearly related to PSII catalysed electron transport [28] and thus may be used in spinach thylakoids to accurately gauge PSII photoinhibition. It can be seen, in agreement with our previous report where the analysis was limited to the initial stages of photoinhibition, that lowering of the excited state population in PSII antenna brought about only in a minor protective effect over the entire photoinhibition process. Owing to the long time scale of these experiments, the difference between the relative excited state levels in control and quencher treated samples, as indicated by the F_M determined during light treatment, varied considerably (Figs. 1 and 2) due to light induced quenching associated with photoinhibition. Comparison between control and samples treated with the singlet quencher is therefore best performed by integration over the relevant time window (Fig. 1B).

In Fig. 2 immunoblot data are presented for the light induced degradation of the reaction centre protein, D1, for the same samples analysed above for photoinhibition and quenching (Fig. 1). As previously reported by others [3,35], the loss of D1 protein is a delayed phenomenon compared with photoinhibition, displaying an approximate half time of 30 min compared with that for photoinhibition of about 5 min (Fig. 1A). When the measurements were performed in the presence of the singlet quencher, which lowered the excited state levels by 67–40% (Fig. 1B) during the course of the experiment, no apparent difference in D1 degradation, with respect to the controls, could be detected. Also shown for comparison in Fig. 2 are the immunoblots for the Cyt *f*, for which a much less pronounced degradation could be detected, and this only after the D1 protein was almost completely degraded.

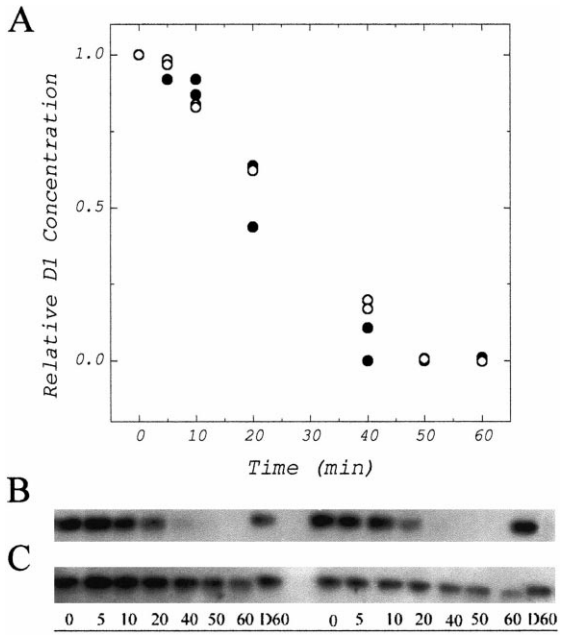

Fig. 2. The kinetics of degradation of the D1 protein. A: Densitometric scans (circles). Open symbols are control samples, closed symbols are samples incubated with 500 μM DNB. B: Immunoblots with antibody raised against D1. C: Immunoblots with antibody raised against Cyt *f*. Samples on the left are controls, and on the right incubated with DNB (500 μM). Also indicated are treatment times in minutes. The samples named D60 are negative controls kept for 60 min in the dark.

4. Discussion

The main result presented in this study is that the light induced degradation of the PSII reaction centre protein D1 is non-significantly modified by reduction of the antenna singlet excited state population by means of an added singlet quencher. This result, together with the limited sensitivity of photoinhibition to antenna excited state levels (Fig. 1, [27–29,36]) demonstrates that the entire process of photoinhibition and its repair, of which D1 degradation is thought to be one of the first steps, displays, at the most, limited sensitivity to the antenna excited state population in isolated thylakoids. We would emphasise that while the present results were obtained with high light fluence, this limited sensitivity of photoinhibition to the antenna excited state population also occurs at very low fluence [28]. From the experiments presented here and previous reports [3,35] it is apparent that in thylakoids the degradation of the D1 protein follows the loss of photochemical activity. It has been argued that when the acceptor side of PSII is 'overreduced' the P_{680} recombination triplet is formed and it is this unquenched triplet which leads to the degradation of closely located proteins, via $^1O_2$ formation [37]. The present results however are not in agreement with this as the antenna excited state quenching is significant over the entire time window investigated and this should have brought about a proportional reduction of the P_{680} triplet. Thus these data point towards a significant role of poorly coupled or completely uncoupled Chls, via the formation of unquenched triplets and hence $^1O_2$ generation. The details of the molecular mechanism which leads to the triggering of D1 degradation are as yet unknown, though the involvement of a conformational change at the level of the Q_B binding pocket [38] and oxidation of side chain amino acidic residues [39] mediated by $^1O_2$, has been suggested. If the trigger is some aspect of photoinhibitory damage itself then the present result is not really surprising in the light of previous studies on photoinhibition [27–29,36]. If, on the other hand, its triggering mechanism is independent of photoinhibition, the present results indicate that uncoupled Chls are probably also involved.

The present results which demonstrate a limited protective effect of added singlet quenchers to both photoinhibition and one of its early repair steps have important implications for the role of the well known non-photochemical quenching phenomenon [40,41]. This process, thought to involve the xanthophyll cycle [41], is widely considered to be an important protective mechanism against photoinhibitory damage by down regulating the excited state population in PSII antenna. The quenching centres seem to be principally located in the Chl *a*/*b* complexes of the external antenna [41,42]. The present paper, together with previous results from this and other laboratories [27–29,36], indicates that the lowering of the excited state population, in itself, is not particularly effective in protection against photoinhibition and the initial stages of repair (D1 degradation). Thus it is important to know whether the lowering of the level of excited states by added singlet quenchers is equivalent to that achieved by the endogenous process i.e. whether the physical location of the quencher is important in determining its protective properties. Singlet quenchers display a similar quenching efficiency in all the isolated PSII complexes (unpublished data), thus it is reasonable to assume that in the photosynthetic membrane they will partition into all complexes. On the other hand the endogenous non-photochemical quenchers are largely located in the Chl *a*/*b* proteins of the external antenna. To appreciate the importance of this difference it is necessary to understand some aspects of energy transfer from the antenna to the photochemical trap in PSII. Two extreme cases may be distinguished. (i) The trap limited case. If PSII were trap limited, with excited states visiting all Chls, including P_{680}, a number of times before photochemical trapping occurs [43], then it is expected that all quenching

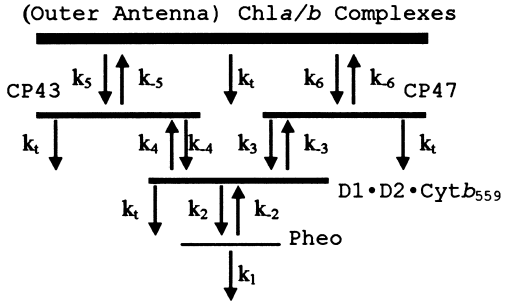

Fig. 3. Antenna–reaction centre PSII model [47]. The Chl *a*/*b* proteins of the external antenna are placed together in one state. The antenna kinetic constant ($k_{\pm3}$, $k_{\pm4}$, $k_{\pm5}$, $k_{\pm6}$) describes the partial diffusion limited trapping necessary to describe the measured low fluorescence yield on the core complexes. In this case calculations were performed for F_M with an F_V/F_M ratio of 0.77. To this end the kinetic constants k_1, k_2 and k_{-2} were modified with respect to the published model which describes fluorescence yield at open reaction centres (F_o). The kinetic constants, in ns^{-1}, are $k_1 = 0.2$, $k_2 = 10$, $k_{-2} = 1.2$, k_3 56, k_{-3} 175, k_4 5.6, k_{-4} 15.9, k_5 40, k_{-5} 184, k_6 20, k_{-6} 83. Each Chl/protein level has a trivial decay rate (k_t) which is 0.5 ns^{-1}. To simulate antenna quenching it is the k_t which is varied.

phenomena will be equivalent, with the entire antenna system being quenched to the same extent irrespective of where the quencher is physically located. In this case added singlet quenchers and endogenous non-photochemical in the external antenna complexes are expected to be equivalent. (ii) The diffusion limited case. In the diffusion limit the rate of energy transfer from the antenna to the trap is the kinetically limiting process in photochemical trapping. As energy flow within PSII antenna complexes is on a femto/picosecond time scale [44–46] diffusion limitation could occur due to energy flow between complexes. In this case it is possible that the location of the quencher is of importance. Recent studies suggest that PSII is a mixture of these two extreme cases with diffusion of the excited states to the reaction centres exercising a kinetic limitation of about 30% [47]. In order to see whether the quencher location is of importance in PSII quenching in such a situation we have performed calculations with the same five state PSII model, recently proposed and which incorporates this antenna kinetic limitation [47]. The model and the kinetic constants are presented in Fig. 3 which describes the F_M level (see [47] for the F_o level). The changes necessary to simulate trap closure with an $F_V/F_M = 0.77$ are given in Fig. 3. The initial excited state population was distributed between the complexes according to their absorption weighting in PSII [48]. In order to simulate a specific quenching located in the Chl *a*/*b* complexes of the external antenna the rate constant for the trivial decay processes (k_t) was increased from 0.5 ns^{-1} to 1.5 ns^{-1}. Results show that all complexes were quenched by 0.53 except the D1/D2/Cyt b_{559} complex which was quenched by 0.52. When, instead, the quencher was uniformly diffused over all antenna complexes, to simulate the singlet quencher situation, the k_t value was 1.2 ns^{-1} and this yielded a quenching of 0.54 on all complexes. Thus we conclude from model calculations, with a realistic PSII model, that there should be no appreciable difference between excited state quenching by added singlet quenchers and endogenous non-photochemical quenching which furthermore suggests that the importance attributed to this latter process as a photoprotective mechanism may need to be reexamined.

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