Free Radical Scavengers Inhibit Light-Dependent Degradation of the 32 kDa Photosystem II Reaction Center Protein

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Involvement of oxygen-free radicals in the rapid, light-dependent degradation of the 32 kDa photosystem II reaction center protein was investigated. The free radical scavengers propyl-gallate and uric acid inhibited 32 kDa protein degradation without affecting linear electron flow. The involvement of singlet oxygen was excluded. Protection from degradation was also afforded under ultra-violet and far-red radiations. These data implicate free-radical damage as a common step in the degradation process, and emphasize the oxygen environment as a causative factor in destabilization of the 32 kDa protein.

Introduction

The crystallization and structural solution of the non-oxygenic bacterial reaction center [1, 2] revealed that homologous domains of the L and M subunits make up the principal binding sites for the 'special pair' donor chlorophylls, the non-heme Fe, and the quinones. Subsequently, characterization of the oxygenic photosystem II (PS II) reaction center from higher plants [3–5] supported models hypothesizing organizational and functional similarities with the non-oxygenic bacterial reaction center (for review see [6, 7]). Similarities extend to pigment orientation, arrangement of the transmembrane helices, amino acid homologies in functional regions, and conservation of the binding sites for quinones and a non-heme Fe. The PS II reaction center is currently thought to consist of five polypeptides, the 32 kDa protein (also called D 1), D 2 (30 kDa), two subunits of cytochrome b_{559} (9 and 4 kDa) and a 4.8 kDa protein [3, 4, 8]. The 32 kDa and D 2 proteins are, respectively, analogous to the L and M subunits of the bacterial reaction center, and are considered to form the nucleus of the PS II reaction center [9].

A major difference between the PS II and bacterial reaction centers is the unique light-dependent turnover of the 32 kDa PS II protein [6, 7]. A phylogenetically conserved cleavage domain (*ca.* from arg238 and ile248), and an α-helix destabilizing region (from arg225 to arg238), have been proposed as determinants of its degradation [6, 7]. Interestingly, these two regions are not conserved in the analogous L subunit of the non-oxygenic bacterial reaction center (see [7, 10]). This has led to the proposal that the rapid turnover of the 32 kDa protein coevolved with PS II in oxygenic photosynthetic organisms [7]. Degradation of the 32 kDa protein occurs over a broad spectral range – ultra-violet (UV), visible and far-red [11–13], and is inhibited by the herbicides DCMU and atrazine [14]. Two separate photosensitizers, one active in the UV and the other in visible and far-red radiation, seem to be involved [12]. However, the degradation of the protein at all these radiations produces a common primary breakdown product [13], suggesting the existence of a common degradation pathway.

The function and mechanism of rapid degradation of the 32 kDa protein are not known. It has been proposed that the semiquinone anion radical formed during the reduction of quinone (Q_B) peri-

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odically reacts to cause 32 kDa protein turnover presumably *via* an active oxygen intermediate [12, 15, 16]. We have tested this possibility and provide here indirect evidence implicating oxygen-free radicals in the initial damage to the 32 kDa protein in the intact plant. Further, we show that the *in vivo* protection of 32 kDa protein degradation by free-radical scavengers occurs without affecting PS II electron flow.

Materials and Methods

Plant material

Axenic cultures of *Spirodela oligorrhiza* were grown phototrophically (15 μmol m^{-2} s^{-1}, cool white fluorescent light, 25 °C) in mineral medium containing 0.5% sucrose as previously described [17].

In vivo fluorescence induction kinetics

Fluorescence of intact plants was measured in the absence and presence of the effectors mentioned in the text using a plant productivity fluorimeter (Model SF 20, Richard Brancher Res. Ltd., Ottawa) connected to an Apple IIe computer. A minimum of three measurements on at least three separate plants were made. The plants were dark-adapted for 30 min prior to analysis in the light. Fluorescence induction kinetics were analyzed and plotted as relative PS II electron transport using the formula $F_{max}-F_{tm}/(F_{max}-F_o)_{control}$, which reflects the effect of the test compound on the ability of PS II to transfer electrons to the plastoquinone pool as previously described [18]. The time *tm* was arbitrarily chosen so as to minimize the fluorescence yield in the control samples and to maximize it in the totally inhibited samples [18].

Pulse-chase experiments and quantification of half-life ($t_{1/2}$)

Spirodela plants, incubated overnight in mineral medium without sucrose, were radiolabeled with 50 μCi/ml of [^{35}S]methionine (New England Nuclear/DuPont, spec. activity 41.96 TBq/mmol) for 3 h in visible light (20 μmol m^{-2} s^{-1}). Following radiolabeling, the plants were washed and transferred to mineral medium containing non-radioactive 1 mM methionine with and without the effectors mentioned in the text. In some experiments, light quality was varied during the chase period as indicated in the legend to Table I. The chase times were 4, 8, 14 and 18 h. Plants were homogenized, membrane proteins fractionated by SDS/PAGE, and gels fluorographed as previously described [19].

Several replicates of each experiment were analyzed by densitometric scanning (LKB, 2202 Ultroscan) of the X-ray films. In all cases the values were determined from responses in the linear range of the films exposed for different times. Values for peak area were normalized with respect to the 26 kDa light harvesting chlorophyll *a*/*b* apoprotein (LHCP), which is a stable protein under the experimental conditions used. Half-life of the 32 kDa protein was calculated from semi-log plots of time *versus* 32 kDa peak area/LHCP peak area as previously described [12].

Results

Free-radical scavengers inhibit 32 kDa protein degradation without affecting fluorescence induction kinetics

Herbicides such as DCMU (diuron) or atrazine are thought to act by binding to the 32 kDa protein and displacing Q_B from its pocket [22, 23]. Under these conditions, linear electron flow and degradation of the 32 kDa protein are both inhibited [6, 14]. This has led to the suggestion that impairment of electron transport is one way of affecting 32 kDa protein degradation. Changes in fluorescence induction kinetics are a good measure of the alterations in the redox state of Q_A, the primary quinone electron acceptor in PS II [20, 21]. Thus, when Q_A oxidation is impeded due to a block in the transfer of electrons to Q_B, the variable fluorescence component shows a more rapid rise that reflects a decrease in the relative PS II electron transport ability [18]. Since our intent was to screen for chemicals that inhibited 32 kDa protein degradation without affecting electron transport, we used fluorescence induction measurements initially to select for such chemicals.

Fig. 1 presents results on the effect of known free-radical scavengers, *viz.*, propylgallate and uric acid [24–26] and the PS II inhibitor DCMU on the fluorescence induction kinetics of intact *Spirodela* plants. As previously established, DCMU caused a decrease in the relative PS II electron

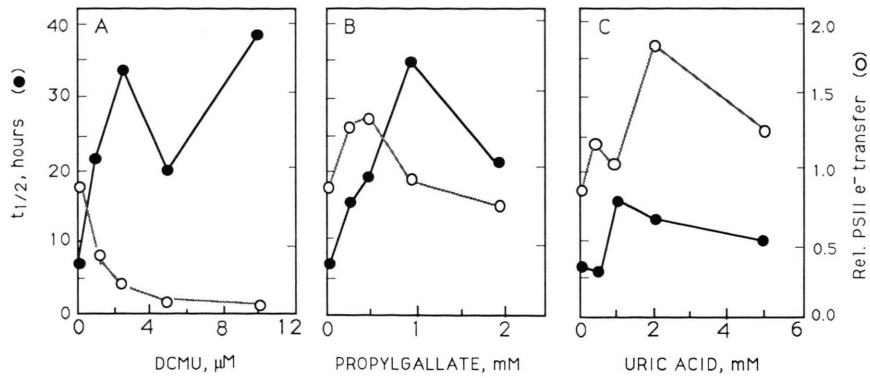

Fig. 1. Effects of DCMU (diuron) and free-radical scavengers, propylgallate and uric acid, on relative PS II electron transport (open circles) and half-life of the 32 kDa protein (closed circles). Fluorescence induction kinetics of intact *Spirodela* plants were followed *in vivo* in the absence and presence of the indicated concentrations of either DCMU (A), propylgallate (B) or uric acid (C) as described in the Materials and Methods section. These data were analyzed as previously described [18] and are plotted as relative PS II electron transport. For the determination of half-life of the 32 kDa protein, pulse-chase experiments were carried out in visible light as described in the Materials and Methods section. Half-life ($t_{1/2}$) was calculated from semi-log plots of time *versus* 32 kDa peak area/LHCP peak area. The data plotted are averages of four independent experiments that were analyzed.

transport ability as compared to the control in a concentration-dependent manner (Fig. 1 A, open circles). Conversely, at low concentrations of propylgallate (0.25–0.5 mM) and uric acid (2 mM) there was actually a perceptible increase in the relative PS II electron transport ability, indicating tighter interaction of the PS II reaction center with the other electron transport chain components, and thus more efficient electron transfer (Fig. 1 B and 1 C, open circles). At higher concentrations, neither propylgallate nor uric acid altered the relative PS II electron transport as compared to the controls. From these data it became apparent that the action of these two chemicals differed from that of DCMU.

The efficacy of propylgallate, uric acid and DCMU on light-dependent degradation of the 32 kDa protein was examined *in vivo* using pulse-chase experiments. Plants were radiolabeled with [^{35}S]methionine for 3 h, the radioactivity was then chased for different time periods in non-radioactive medium and thylakoid proteins were fractionated by SDS-PAGE. The quantified data, presented in Fig. 1 (closed circles), show the effect of varying concentrations of propylgallate, uric acid and DCMU on the half-life of 32 kDa protein in visible light. As demonstrated previously [14], DCMU was very effective in inhibiting the rate of degradation of the 32 kDa protein (Fig. 1 A, closed circles); the half-life of the protein was markedly increased from about 7 h (at 0 mM) to about 34 h (at 2.5 mM), coinciding well with the concentration-dependent decrease in the relative PS II electron transport (Fig. 1 A, open circles).

Propylgallate and uric acid also inhibited degradation of the 32 kDa protein. Of the two free-radical scavengers, propylgallate was the better inhibitor (compare Fig. 1 B to Fig. 1 C, closed circles), producing a level of protection similar in degree to that of DCMU. In contrast to the DCMU effect on fluorescence induction kinetics, however, the concentrations of propylgallate and uric acid that markedly increased the half-life of the 32 kDa protein did not alter the electron transport properties of PS II in intact plants (compare Fig. 1 B, C to Fig. 1 A). A perusal of the Coomassie-stained gels revealed no major change in the steady-state protein profiles in the presence of these free radical scavengers (data not shown), indicating the specificity of their effect on 32 kDa protein degradation. These data suggest the involvement of oxygen-free radicals in the degradation of the 32 kDa PS II protein.

Propylgallate is effective in visible, ultra-violet and far-red radiation

Degradation of the 32 kDa protein occurs also in UV and far-red radiation as well as in visible

light [11–13]. As DCMU inhibits degradation of the protein in all these light regimes it has been suggested that a common pathway is involved. If oxygen-free radicals cause damage to the protein and prime it for the degradation process, we hypothesized that propylgallate should prevent damage to the protein and, thus, inhibit its degradation in UV and far-red radiations as well as it did in the visible light. Indeed, propylgallate was as effective as DCMU at inhibiting 32 kDa protein degradation in these light regimes (Table I). The triazine herbicide atrazine, which also binds to the 32 kDa protein, also inhibited its degradation at the three spectral regions, the values (at 10 μM atrazine) being very similar to those obtained with propylgallate (Table I). These data implicate a common priming event that targets the 32 kDa protein for degradation and are in line with the involvement of oxygen-free radicals in this process.

Possible species in the oxidative damage to the 32 kDa protein

The presence of chlorophyll molecules in the vicinity of the water oxidation complex of PS II makes the chloroplast a potential source for generating singlet oxygen (for review see [27]). There are dependable diagnostic tests for the involvement of singlet oxygen in biological processes. For instance, it is possible to stabilize singlet oxygen in D_2O and thereby promote its deleterious effects [28], or to scavenge singlet oxygen by quenchers such as selenomethionine thereby inhibiting its effects [29]. Both these tests have been successfully used to implicate singlet oxygen in chemical-induced senescence of *Spirodela* plants [30]. Results, summarized in Table II, show that instead of promoting photodestruction of the 32 kDa protein, as would have been expected if singlet oxygen was involved, D_2O, if anything, slowed down protein degradation. Likewise, the singlet oxygen quencher selenomethionine was ineffective. These data rule out the involvement of singlet oxygen in the degradation of the 32 kDa protein.

Table I. Inhibition of 32 kDa protein degradation by DCMU and propylgallate. Plants were pulse-labeled for 3 h in visible light and radioactivity chased for different times in the absence or presence of either 10 μM DCMU, 10 μM atrazine or 0.5 mM propylgallate as previously described [14]. Light conditions for the chase phase were: visible light (cool white fluorescent, 6 μmol m^{-2} s^{-1}), UV light (300 nm, Rayonet Photoreactor Bulb, Southern New England UV Co., 6 μmol m^{-2} s^{-1}), far-red (700–730 nm, 6 μmol m^{-2} s^{-1}) with filter as previously described [11]. Membrane proteins were fractionated by SDS-PAGE [19]. Gels were loaded on an equal protein basis and visualized by fluorography. Rates of degradation were determined as described in the Materials and Methods section (plus and minus the inhibitor). Degree of inhibition is expressed as % ± standard error of the mean (*n* ranged from 4 to 15). Percent inhibition was calculated individually for each time point from the rate of degradation ± the inhibitor. The inhibition values were then averaged.

Light	% Inhibition of control		
	DCMU	Atrazine	Propylgallate
UV	72 ± 4	57 ± 3	57 ± 4
Visible	85 ± 6	65 ± 8	64 ± 10
Far-red	64 ± 16	69 ± 17	66 ± 18

Table II. Effect of D_2O and selenomethionine on the degradation of the 32 kDa protein. Half-life of the 32 kDa protein in the absence and presence of the indicated concentrations of D_2O and selenomethionine was determined as described in the Materials and Methods section and in the legend to Table I. *n* denotes the number of experiments analyzed.

Effector	Half-life [h]	*n*
D_2O (%)		
0 (control)	8.5 ± 2.2	6
20	8.6 ± 1.6	6
40	9.6 ± 2.72	6
60	10.65 ± 3.41	7
80	12.26 ± 2.6	6
Selenomethionine (mM)		
0 (control)	9.7 ± 2.3	7
0.5	10.71 ± 3.17	7
2.0	9.22 ± 1.94	7

Discussion

Free radical-mediated oxidative damage of proteins and their subsequent proteolysis has been explored in several eukaryotic systems. There is mounting evidence that radical fluxes may enhance protein turnover, mostly in mammalian cells [31, 32]. Our data indirectly implicate oxygen-free radicals in the 32 kDa protein degradation. The nature of the oxygen radical species involved was not determined. However, both of the radical scavengers, *viz.*, propylgallate and uric acid, that were effective in inhibiting 32 kDa protein degradation are water-soluble and have been reported to be

more specific for hydroxy radicals [33, 34]. Based on these observations, and the data on the non-involvement of singlet oxygen, it is a reasonable assumption that hydroxy radicals may cause the damage to 32 kDa protein that results in its degradation. However, we cannot rule out the possibility that other oxygen radicals such as superoxide anion or peroxy radicals may also be involved since uric acid scavenges both the hydroxy and peroxy radicals [35, 36].

Many antioxidants stabilize proteins by complexing with metal ions such as copper and iron, thereby keeping these metal ions from participation in radical generation. Uric acid and propylgallate are not only good scavengers of reactive oxygen radicals, but also known metal chelators [37]. In this context, it is noted that iron is a central binding ligand in the PS II reaction center, being in close vicinity to the degradation domain of the 32 kDa protein [6, 7]. If the scission of the 32 kDa protein involves non-heme iron, then the protective effect of uric acid and propylgallate may be to sequester the metal and thus prevent damage to the protein.

Semiquinone anion radical formed during the reduction of Q_B has been implicated as a photosensitizer in the photodestruction of the 32 kDa protein [12]. This radical can readily react with oxygen [38]. As a result, the quinone could be forced out of the binding site. An empty quinone-binding pocket has been postulated [39] to result in a conformational change around the α-helix destabilizing and cleavage regions (see [6, 7]), allowing better access to the protease. According to this postulate, herbicides such as DCMU and atrazine would replace the quinone in the protein, prevent the formation of protease-sensitive conformation and thus stabilize it. However, this postulate does not take into consideration the fact that, in the dark, 50% of the quinone-binding sites are empty [40], and yet the protein is stable [14]. On the other hand, Critchley [40] has argued that the quinone must be in place on the protein for degradation to occur. Further experimentation is required to distinguish between the various degradation schemes proposed. In either situation, the inhibition of 32 kDa protein degradation by propylgallate and uric acid needs to be taken into consideration. It should be noted that the semiquinone anion radical is formed during electron flow through either the rapidly-degraded 32 kDa protein in PS II or the relatively stable L subunit, its counterpart in the photosynthetic bacterial reaction center. Thus, this radical species, while possibly necessary, cannot be a sufficient determinant for 32 kDa protein degradation. PS II organisms split water in an oxygenated environment while as purple bacteria use hydrogen donors as sources of electrons in an anaerobic environment (see [6, 7]). It would seem that preventing interaction of oxygen with the 32 kDa protein (by using effective free-radical scavengers) can stabilize the protein. The demonstration here of the involvement of oxygen radicals in the degradation of the 32 kDa protein emphasizes the oxygen environment as a causative factor for destabilizing this PS II reaction center component.

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