Covalent dimerization of ribulose biphosphate carboxylase subunits by UV radiation

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The effect of UV radiation (UV-A, UV-B and UV-C) on ribulose biphosphate carboxylase from a variety of plant species was examined. The exposition of plant leaves or the pure enzyme to UV radiation produced a UV-dependent accumulation of a 65 kDa polypeptide (P65). Different approaches were utilized to elucidate the origin and structure of P65: electrophoretic and fluorographic analyses of ³⁵S-labelled ribulose biphosphate carboxylase exposed to UV radiation and immunological experiments using antibodies specific for P65, for the large and small subunits of ribulose biphosphate carboxylase and for high-molecular-mass aggregates of the enzyme. These studies revealed that P65 is a dimer, formed by the covalent, non-disulphide linkage of one small subunit with one large subunit of ribulose biphosphate carboxylase. For short periods of time (< 1 h), the amount of P65 formed increased with the duration of the exposure to the UV radiation and with the energy of the radiation

applied. Prolonged exposure to UV radiation (1–6 h) resulted in the formation of high-molecular-mass aggregates of ribulose biphosphate carboxylase. Formation of P65 was shown to depend on the native state of the protein, was stimulated by inhibitors of enzyme activity, and was inhibited by activators of enzyme activity. A UV-independent accumulation of P65 was also achieved by the *in vitro* incubation of plant crude extracts. However, the UV-dependent and the UV-independent formation of P65 seemed to occur by distinct molecular mechanisms. The UV-dependent accumulation of P65 was immunologically detected in all species examined, including *Lemna minor*, *Arum italicum*, *Brassica oleracea*, *Triticum aestivum*, *Zea mays*, *Pisum sativum* and *Phaseolus vulgaris*, suggesting that it may constitute a universal response to UV radiation, common to all photosynthetic tissues.

INTRODUCTION

There is increasing concern about the higher levels of UV-B radiation reaching the Earth's surface because of stratospheric ozone depletion [1]. Although UV-B represents only a minute fraction of the entire electromagnetic spectrum that reaches the surface of the planet, the observed increase may have profound photobiological effects due to its absorption by proteins and nucleic acids [2]. These observations have resulted in a large number of studies on the effect of the various types of UV radiation (UV-A, 320–400 nm; UV-B, 290–320 nm; UV-C, 200–290 nm) on living systems. UV-C is absent in daylight and is not relevant for living organisms in the natural environment because it does not reach the Earth's surface even in the event of severe ozone depletion. UV-A is not absorbed by the ozone layer, being present in large amounts in normal sunlight. Nevertheless, the three types of UV radiation produce, in some cases, similar responses in living organisms.

There are two potential primary lesions involved in UV-B-induced cellular damage that effectively inhibit many plant processes: the formation of DNA photoproducts that interfere with DNA replication and transcription [3], and the photochemical modification of proteins and other molecules, particularly those associated with membranes, which reduces enzyme activity and may sensitize subsequent photochemical reactions [4–6]. As a result, UV-B radiation causes a multitude of physiological and biochemical changes in plants, such as inhibition of

photosynthesis [7], modification of pigment composition [8] and disruption of membrane structure [9], that ultimately lead to a severe deleterious effect on plant growth and development [10,11]. In particular, the effect of UV radiation on photosynthesis has been the subject of several investigations. However, the site and mechanism of action of UV-B photoinhibition are not fully understood. It has been established that photosynthetic capacity may be reduced (i) by a direct effect of the radiation on the primary photochemical reactions and on the Calvin cycle enzymes, or (ii) indirectly by affecting photosynthetic pigments and stomatal function [12,13].

Little is known about the effect of UV radiation on plant ribulose biphosphate carboxylase (RuBP carboxylase, EC 4.1.1.39), the most abundant protein in the world [14]. This enzyme catalyses the initial reactions of the photosynthetic and photorespiratory pathways [15], and is composed of eight large and eight small subunits, with molecular masses of 52 kDa and 14.5 kDa, respectively, in the case of *Lemna minor* [16]. A number of studies, performed with pea, soybean and cucumber plants exposed to UV-B radiation, reported a decrease in the amount and a change in the catalytic properties of RuBP carboxylase [6,17]. Huang et al. [18] postulated that a rapid degradation of the enzyme occurs under such conditions. In this respect, it has been suggested that the severe UV-induced inhibition of photosynthetic CO₂ uptake may be primarily due to changes in the carboxylase reaction [19].

In the present work we observed a decrease in RuBP carb-

Abbreviations used: DTT, dithiothreitol; LSU, large subunit of RuBP carboxylase; P65, 65 kDa polypeptide; RuBP carboxylase, ribulose biphosphate carboxylase; SSU, small subunit of RuBP carboxylase.

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oxylase amount and activity detected when plants are exposed to UV-A, UV-B or UV-C radiation, concomitant with the accumulation of a 65 kDa polypeptide (P65). Our results indicate that this polypeptide, formed by the covalent linkage of one large and one small subunit of RuBP carboxylase, is produced by the effect of the radiation on the enzyme molecule.

MATERIALS AND METHODS

Plant material and growth conditions

Lemna minor L. was grown autotrophically under sterile conditions, at 25 °C under continuous light, in a complete culture medium as described previously [20]. Seeds of wheat (*Triticum aestivum* L.), corn (*Zea mays* L.), pea (*Pisum sativum* L.), kidney bean (*Phaseolus vulgaris* L.) and cabbage (*Brassica oleracea* L.) were sown in sand and grown at 25 ± 1 °C in a 16 h/8 h light/dark cycle under fluorescent lighting for up to 15 days. The *Arum italicum* Miller plants were collected from a local farm and incubated under the conditions described for wheat. The plants were watered as required.

Chemicals

2-Carboxy-D-ribitol 1,5-bisphosphate and 2-carboxy-D-arabinitol 1,5-bisphosphate were synthesized essentially by the method described by Collatz et al. [21]. Briefly, D-ribulose 1,5-bisphosphate was incubated in the presence of KCN (1:1.6, w/w) in 20 mM Tris/HCl buffer, pH 8.5, for 16 h at room temperature. The reaction was stopped by the addition of formic acid (1 % final) and taken to dryness under vacuum on a Speedvac. The residue was washed in 1 % formic acid, taken to dryness, dissolved in 20 mM Tris/HCl buffer, pH 9.0, and incubated for 24 h at room temperature to ensure that no lactone forms were present. The pH of the solution was finally adjusted to 8.0. All other biochemicals and general laboratory chemicals were reagent grade or better. All pH adjustments were performed at room temperature and 'Milli-Q plus' water (Millipore, France) was used throughout.

UV treatments

Three different systems were exposed for different periods of time to UV radiation: (1) fronds of *L. minor*, incubated in complete growth medium in the bottom dish of Petri dishes; (2) the seedlings of other plant species and the plants of *A. italicum*, after a thorough watering, in pots containing soil; and (3) a solution of purified *Lemna* RuBP carboxylase (800 µg/ml) in

100 mM Tris/HCl buffer, pH 7.5, contained inside a 3 ml silica cuvette, with gentle agitation. Plant leaves were exposed uncovered to the UV radiation. UV radiation was supplied by fluorescent lamps (Philips TLD 15W/05 for UV-A; Vilber-Lourmat T-15M for UV-B; Philips TUV 15W G15 T8 for UV-C) suspended 60 cm above the various systems. The data presented in Table 1 show the intensity of the different types of UV radiation employed. For comparative purposes, data are also included about the UV radiation reaching *L. minor* cultures incubated under normal growth conditions. It is important to mention the presence of a glass plate in the growth chamber, between fluorescent lamps and *L. minor*. After the exposure, plant or protein samples were immediately frozen in liquid nitrogen and stored at -70 °C until needed.

In vivo labelling

Plants of *L. minor* were placed in Petri dishes containing complete growth medium and incubated for 60 min, under normal illumination or UV radiation, in the presence of L-[³⁵S]methionine (20 kBq/ml; Amersham, U.K.). The plants were harvested, washed with water containing unlabelled methionine (1 mM), frozen in liquid nitrogen and stored at -70 °C.

Extraction of protein

In the standard extraction procedure, the plant tissues were frozen in liquid nitrogen, ground to a fine powder and extracted (2.5 ml per g fresh weight) in 100 mM Tris/HCl buffer, pH 7.5, containing 1 mM PMSF. The homogenate was filtered through two layers of cheesecloth and clarified by centrifugation at 30000 g for 30 min at 2 °C. The supernatant (crude extract) was directly utilized for the UV-independent accumulation of P65 or desalted at 2 °C on a PD-10 prepacked Sephadex G-25M column (9.1 ml bed volume, 2.5 ml sample; Pharmacia/LKB, Sweden), previously equilibrated with 20 mM Tris/HCl buffer, pH 7.5.

Where appropriate, two extraction procedures were utilized under denaturing conditions: (1) extraction of the plant tissue in 80 mM Tris/HCl buffer, pH 6.8, containing 3 % (w/v) SDS and 1.4 % (v/v) 2-mercaptoethanol, as described by Granier [22]; (2) extraction of the plant tissue in water-saturated phenol, as described by Monroy et al. [23].

Purification of *Lemna* RuBP carboxylase

The desalted extract was loaded onto the Mono Q HR5/5 anion-exchange column of the fast protein liquid chromatography system (FPLC, Pharmacia/LKB), previously equilibrated with 20 mM Tris/HCl buffer, pH 7.5. The bound proteins were eluted with a gradient of NaCl (0–1 M). The A₂₈₀ peak corresponding to RuBP carboxylase, identified as described by Ferreira and Teixeira [20], was eluted with approx. 0.3 M NaCl, desalted on a PD-10 column and concentrated on the Mono Q column, equilibrated in the same buffer. The concentrated enzyme was then loaded onto the Superose 6 HR10/30 gel-filtration column of the FPLC, previously equilibrated with 100 mM Tris/HCl buffer, pH 7.5. The flow rate was 0.5 ml/min and 0.5 ml fractions were collected. The fractions containing RuBP carboxylase were shown to be free of protein contaminants by SDS/PAGE.

Assay of RuBP carboxylase activity

RuBP carboxylase was fully activated and the enzyme carboxylase activity assayed essentially as described by Lorimer et

Table 1 Intensity of the UV radiation employed

Fronds of *L. minor*, incubated in complete growth medium, the plants of *A. italicum*, the seedlings of other plant species and the solutions of purified RuBP carboxylase were subjected to UV treatments as described in the Materials and methods section. UV irradiation was measured with a Vilber-Lourmat radiometer VLX-3W (Marne la Vallée, France), using an appropriate sensor (CX-365 for UV-A, CX-312 for UV-B and CX-254 for UV-C).

Type of UV radiation	Irradiation reaching the plants (W·m ⁻²)	
	Under normal growth conditions (<i>Lemna</i> growth chamber)	During UV treatment
UV-A	0.27	0.27
UV-B	0.08	1.05
UV-C	0.02	2.10

al. [24]. Activation was achieved by incubation of the enzyme in 100 mM Tris/HCl buffer, pH 8.4, containing 10 mM MgCl_2 , 10 mM NaHCO_3 and 5 mM dithiothreitol (DTT), for 45 min at 35 °C. The enzyme activity was measured in a 100 mM glycylglycine buffer, pH 8.0, containing 20 mM MgCl_2 , 2 mM DTT, 10 mM $\text{NaH}^{14}\text{CO}_3$ (3.7 MBq/mmol) and 1.2 mM D-ribulose 1,5-bisphosphate in a 250 μl volume at 25 °C. The reaction was stopped after 30 s by addition of 0.2 ml of 2 M HCl. The contents of the vials were evaporated to dryness in a freeze-dryer and dissolved in water. Radioactive samples were counted for ^{14}C in a quench calibrated LS 3801 liquid scintillation counter (Beckman, U.S.A.). Ready-safe (Beckman) was used as the scintillation cocktail.

Protein was measured by a modification of the Lowry method [25].

Electrophoresis, autoradiography and immunoblot analysis

All protein samples were boiled for 3 min in the presence of 2% (w/v) SDS and 0.1 M 2-mercaptoethanol and subjected to SDS/PAGE in 12.5% (w/v) acrylamide slab gels, essentially as described by Laemmli [26], except that *m*-Cresol Purple was used as the tracking dye and 0.1 M sodium acetate was included in the anode buffer to resolve polypeptides with molecular masses ranging from 2.5 kDa to greater than 200 kDa [27]. Polypeptides in gels were fixed with 12% (w/v) trichloroacetic acid and stained with Coomassie Brilliant Blue R-250. The large and small subunits of RuBP carboxylase (LSU and SSU, respectively) were identified in SDS/PAGE gels as described previously [16]. When appropriate, the gels were stained with a copper solution to visualize the polypeptides [28,29]. In brief, the gels were dipped for several seconds in water, immersed in a solution containing 0.3 M CuCl_2 , incubated for 5 min with gentle agitation, washed for 2–3 min in water to remove excess reagent and stored in water until needed. The acrylamide bands containing the proteins were sliced and destained and the polypeptides eluted and re-subjected to SDS/PAGE [28,29]. The molecular-mass markers used were: rabbit muscle myosin (subunit, 205 kDa); *Escherichia coli* β -galactosidase (subunit, 116 kDa); bovine plasma albumin (66 kDa); egg albumin (45 kDa); bovine erythrocyte carbonic anhydrase (29 kDa); and bovine milk α -lactalbumin (14.2 kDa).

Polypeptides separated by SDS/PAGE were blotted on to a nitrocellulose membrane using a semi-dry transfer cell (Bio-Rad, U.S.A.), essentially as described by Towbin et al. [30]. Just before blotting, the SDS-gel and membrane were equilibrated in cold transfer buffer [25 mM Tris, 0.19 M glycine, 0.1% SDS and 20% (v/v) methanol] for 10–15 min. The blots were then processed using appropriate antibodies by the method described by Ferreira and Shaw [31], except that Tween-20 (0.05%, v/v) was included in the antibody-containing solutions.

In SDS/PAGE gels destined for fluorography, the volume of sample loaded in each well was adjusted to contain 9 Bq of ^{35}S -labelled RuBP carboxylase or 36 Bq of ^{35}S -labelled total protein extract. Following separation by SDS/PAGE, the gel was dried and subjected to fluorography as described by Bonner and Laskey [32], using Hyperfilm-MP (Amersham, U.K.). The fluorograms were developed after 5 days of exposure at -70°C .

Quantification of P65, LSU and SSU on SDS-gels

Twelve different amounts of native RuBP carboxylase (ranging from 1 to 60 μg), corresponding to 12 different amounts of SSU (ranging from 0.21 to 12.34 μg) and of LSU (ranging from 0.79 to 47.66 μg), were electrophoresed and the resulting SDS-gels

stained with Coomassie Brilliant Blue and subjected to densitometric analysis using an UltroScan XL densitometer (Pharmacia/LKB). Utilizing regression analysis, we have used a computer program that fits, by the method of least squares, a power curve ($y = ax^b$) to our set of data and calculates the corresponding coefficient of determination (r^2). The following regression equation, valid for RuBP carboxylase subunits, was obtained:

$$y = 23.25x^{0.6546}, r^2 = 0.994$$

where x represents the amount of RuBP carboxylase subunits, expressed in μg , and y represents the area of the corresponding stained protein bands in SDS-gels, expressed in mm^2 .

Preparation of anti-SSU, anti-LSU and anti-P65 antibodies

Specific anti-SSU and anti-LSU antibodies were prepared by purification of *Lemna* RuBP carboxylase and fractionation of the two polypeptides (SSU and LSU, respectively) by preparative SDS/PAGE. In the case of specific anti-P65 antibodies, a solution containing pure RuBP carboxylase was exposed to UV-B radiation for 90 min prior to SDS/PAGE. After staining with Coomassie Brilliant Blue, the bands corresponding to the three polypeptides (SSU, LSU and P65) were sliced and utilized for immunization. A sample of each antigen was re-subjected to SDS/PAGE to ensure the absence of cross-contamination. The macerated acrylamide bands (each containing approx. 200 μg of P65, 400 μg of LSU or 200 μg of SSU) were mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously into New Zealand female rabbits. To obtain a high titre, three booster injections were given every 2 weeks in complete Freund's adjuvant diluted 1:10 with incomplete adjuvant. At intervals blood was collected from the marginal ear vein and the titre determined by the ELISA technique [33]. Total blood was taken from the heart 9 days after the third booster injection. Blood samples were allowed to clot and the serum was collected and stored frozen at -70°C . Anti-SSU and anti-LSU IgG were purified by affinity chromatography on the FPLC Protein G–Superose HR10/2 column. The antiserum was desalted on a PD-10 column, previously equilibrated in 20 mM sodium phosphate buffer, pH 7.0, and loaded on to the Protein G column, equilibrated in the same buffer. IgG were eluted with 0.1 M glycine/HCl buffer, pH 2.7. Fractions (1 ml) were collected into test tubes containing 44 μl of 1 M Tris/HCl buffer, pH 9.0, to neutralize the IgG solutions.

Preparation of antibodies to high-molecular-mass aggregates of RuBP carboxylase

Lemna fronds were deprived of calcium for 6 days, a condition that is known to produce polymerization of RuBP carboxylase into high-molecular-mass aggregates [20]. These aggregates produce a smear in SDS/PAGE gels, suggesting that they correspond to multiple molecular species with different degrees of aggregation (results not shown). This suggestion is further supported by the observation that after gel filtration, aggregated RuBP carboxylase elutes from the Superose 6 column of the FPLC over a region corresponding to a wide range of molecular masses [20]. Total protein was extracted and the RuBP carboxylase aggregates purified on the FPLC Mono Q HR5/5 column [20] and utilized in the preparation of antibodies as described for SSU, LSU and P65.

Figure 1 UV-B-dependent accumulation of P65

(A) and (B) refer to extracts of irradiated *Lemna* fronds, (C) and (D) to irradiated purified RuBP carboxylase, with exposure times of 0 to 30 min in (A) and (C) and 0 to 6 h in (B) and (D). Samples of 50 μg of *Lemna* total soluble protein or 25 μg of RuBP carboxylase were loaded in each lane.

RESULTS

UV-dependent accumulation of a P65

When plants of *Lemna minor* are exposed to UV-B radiation for various periods of time and their protein pattern analysed by SDS/PAGE, a gradual but steady accumulation of a 65 kDa polypeptide (P65) is clearly observed (Figure 1A). Prolonged exposure results in higher accumulation of the polypeptide so that a large amount is present in the plants after 4 h (Figure 1B). Thereafter, the levels of P65 tend to decline, together with those of the LSU and SSU of RuBP carboxylase, in a way suggesting its conversion into high-molecular-mass aggregates. In this respect, it is important to note the presence of stained material on the top of the stacking and separating gels after 5 and 6 h of exposure (Figure 1B).

In another experiment, *L. minor* plants were exposed to normal illumination or to UV-B radiation and their soluble protein extracted with Tris/HCl buffer, with SDS-containing buffer or with phenol and analysed by SDS/PAGE (results not shown). The accumulation of P65 was observed in all plants exposed to the UV radiation regardless of the extraction medium utilized, suggesting that the detection of this polypeptide does not result from an artefact occurring during the extraction procedures.

The exposure of a solution containing purified RuBP carboxylase (800 $\mu\text{g}/\text{ml}$) to UV-B radiation also resulted in the formation of P65 and, for prolonged periods, in the appearance of high-molecular-mass conjugates (Figures 1C and 1D). However, when compared with *L. minor* (Figures 1A and 1B), the exposure of the pure enzyme leads to a faster rate of P65 accumulation.

To determine whether the UV-dependent accumulation of P65 was due to *de novo* synthesis of polypeptides or to covalent modification of pre-existing proteins, an experiment was performed in which *L. minor*, incubated in the presence of [^{35}S]methionine (0.3 MBq/15 ml) or a solution of purified ^{35}S -labelled RuBP carboxylase (800 $\mu\text{g}/\text{ml}$; 0.46 Bq/ μg of RuBP carboxylase), was exposed to normal illumination or to UV-B radiation for 60 min. Total polypeptides (75 μg of *Lemna* total

Figure 2 Effects of UV-A, UV-B and UV-C on P65, LSU, SSU and RuBP carboxylase activity

(A), (B) and (C) refer to irradiated *Lemna* fronds, (D), (E) and (F) to irradiated purified RuBP carboxylase. Three types of UV radiation were used: UV-C (●), UV-B (■) and UV-A (▲). (A, B, D and E): the amounts of P65, LSU and SSU were determined by SDS/PAGE; 45 μg of total soluble protein (A, B) or 15 μg of RuBP carboxylase (D, E) was loaded in each well. (C and F): 100% of enzyme activity corresponds to 1.0 μmol of CO_2 fixed $\cdot \text{min}^{-1} \cdot \text{g}^{-1}$ fresh weight (C) or 1.6 μmol of CO_2 fixed $\cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ RuBP carboxylase (F).

soluble protein or 20 μg of RuBP carboxylase per lane) and total ^{35}S -polypeptides (36 Bq of ^{35}S -*Lemna* total soluble protein or 9 Bq of ^{35}S -RuBP carboxylase per lane) were analysed by SDS/PAGE and by fluorography, respectively. The results obtained show that the exposure of ^{35}S -RuBP carboxylase to UV-B leads to the accumulation of ^{35}S -P65. However, when the plants are exposed for 1 h to UV-B in the presence of [^{35}S]methionine, a differential result is obtained; P65 is formed but is devoid of radioactivity. The pattern of polypeptides synthesized *de novo* by *L. minor* during 1 h is essentially the same and independent of the illumination conditions. Under these circumstances, no detectable amount of ^{35}S -P65 was formed, indicating that this polypeptide is not synthesized *de novo* but rather results from the UV-B radiation-induced covalent modification of pre-existing, non-radioactive polypeptides. In other words, a 1 h exposure of *L. minor* to UV-B radiation in the presence of [^{35}S]methionine is not enough to allow the synthesis of ^{35}S -labelled native RuBP carboxylase and the subsequent formation, in detectable quantities, of ^{35}S -P65. This result was confirmed by experiments involving protein synthesis inhibitors, namely cycloheximide (an inhibitor of cytoplasmic protein synthesis) and chloramphenicol (an inhibitor of mitochondrion and chloroplast protein syntheses). The plants were incubated, under normal lighting, in growth medium containing cycloheximide (20 or 200 $\mu\text{g}/\text{ml}$) and chloramphenicol (200 or 400 $\mu\text{g}/\text{ml}$) and, after 2 h, exposed to normal illumination or to UV-B radiation. No detectable effect of the protein synthesis inhibitors was observed on the UV-dependent accumulation of P65 (results not shown).

The experiment illustrated in Figure 2 shows the effects of the different types of UV radiation (UV-A, UV-B and UV-C) on RuBP carboxylase activity (Figures 2C and 2F) and on the rate of accumulation of P65 (Figures 2A and 2D), as well as on the

Figure 3 Purification of P65

RuBP carboxylase was purified from *L. minor* grown under normal illumination (A–C) or exposed for 3 h to UV-B radiation (D–F). In either case, the total soluble protein was isolated and fractionated on the Mono Q column of the FPLC (A, D). Native RuBP carboxylase was further purified on the Superose 6 column of the FPLC (B, E). The enzyme was then dissociated into its subunits and subjected to denaturing gel filtration on the Superose 6 column (C, F). Peak 1, native RuBP carboxylase; peak 2, nucleic acids; peaks 3 and 4, LSU and SSU, respectively; peak 5, P65.

rate of disappearance of LSU and SSU (Figures 2B and 2E), obtained upon exposure of *L. minor* or a solution containing purified RuBP carboxylase (800 $\mu\text{g}/\text{ml}$). The main features are: (i) exposure of *Lemna* or the pure enzyme gives similar results, with the exception of a faster response in the latter case; (ii) the rate of accumulation of P65 is higher under UV-C, decreases under UV-B and is particularly low under UV-A radiation; and (iii) the rates of disappearance of LSU and SSU follow closely the rate of P65 formation for the three types of UV radiation. A reduction in enzyme activity occurs, its rate decreasing from UV-C to UV-B and being particularly low for UV-A. As a whole, these results indicate that exposure to UV radiation leads to the accumulation of P65 and the loss of enzyme activity and suggest that P65 is formed by the UV-induced covalent modification of RuBP carboxylase, probably involving the covalent linkage of one LSU (52 kDa) with one SSU (14.5 kDa).

A comparison between Table 1 and Figure 2(A) shows that P65 accumulates when the plants are incubated under UV-A radiation (0.27 $\text{W} \cdot \text{m}^{-2}$) but not when the plants are subjected to UV-A radiation (0.27 $\text{W} \cdot \text{m}^{-2}$) plus photosynthetically active radiation. In this respect, several reports exist that show the dependence of UV radiation effects on the levels of photosynthetically active radiation incident during growth [34–36].

Purification of P65

Plants of *L. minor*, either grown under normal illumination (control) or exposed for 3 h to UV-B radiation, were extracted and the soluble protein isolated and fractionated by anion-exchange chromatography on the Mono Q column of the FPLC (Figures 3A and 3D). The protein peak corresponding to native RuBP carboxylase was, in each case, analysed by SDS/PAGE and found to be composed of two types of polypeptide chains (LSU and SSU) in the case of the control plants, or three types of polypeptide chains (P65, LSU and SSU) in the case of the UV-exposed plants. Native RuBP carboxylase was further purified by gel filtration on the Superose 6 column of the FPLC, with no

detectable differences being observed between the A_{280} peaks corresponding to the two groups of plants (Figures 3B and 3E). The native enzyme was then concentrated on the Mono Q column, dissociated into its subunits by a 30 min incubation at 37 °C, followed by a 3 min incubation at 100 °C, in the presence of 1% (w/v) SDS and 25 mM 2-mercaptoethanol, and subjected to denaturing gel filtration on the Superose 6 column of the FPLC previously equilibrated in buffer containing 1% (w/v) SDS (Figures 3C and 3F). The A_{280} peaks were collected and analysed by SDS/PAGE (results not shown). The results presented in Figure 3 clearly show that P65 co-purifies with RuBP carboxylase, suggesting that it forms an integral part of the native enzyme.

UV-independent accumulation of a 65 kDa polypeptide

The *in vitro* incubation, for 12 h at 25 °C, either in the light or in darkness, of a crude extract prepared from control plants of *L. minor* resulted in the formation of a 65 kDa polypeptide, clearly detected as a major protein band in SDS-gels (see Figure 4A, lane 5). Prolonged periods of incubation resulted in the accumulation of larger amounts of the polypeptide. A similar result was obtained with extracts prepared from wheat leaves. Under these conditions, the 65 kDa polypeptide was clearly visible on Coomassie Brilliant Blue-stained SDS-gels as little as 1 h after the beginning of the incubation period (results not shown). Preliminary experiments have shown that plant crude extracts contain an unidentified, heat- and acid-labile, low-molecular-mass dimerization factor involved in P65 formation and that the UV-independent formation of this polypeptide is inhibited in the presence of inhibitors of stress-induced oxidase systems (KCN + EDTA) or reducing agents (2-mercaptoethanol) (results not shown).

Immunodetection of P65

Four different antibodies, anti-SSU, anti-LSU, anti-P65 and anti-(high-molecular-mass RuBP carboxylase aggregates), were used to detect and characterize the 65 kDa polypeptide formed either in a UV-dependent manner (in a variety of plant species exposed to UV-C radiation or in a solution containing purified *Lemna* RuBP carboxylase), or in a UV-independent manner. For the production of highly specific, non-cross-reacting, anti-SSU, anti-LSU and anti-P65 antibodies, *Lemna* RuBP carboxylase was purified, exposed to UV-B radiation for 90 min and subjected to preparative SDS/PAGE. This procedure ensures the isolation of SSU, LSU and P65 free of cross-contamination. For the preparation of anti-(high-molecular-mass aggregates of RuBP carboxylase), *L. minor* was incubated for 6 days in a growth medium lacking calcium and the conjugates were subsequently purified as described by Ferreira and Teixeira [20].

The experiment illustrated in Figure 4(A) shows the polypeptide patterns of samples containing P65 formed under a variety of conditions, namely *L. minor* or a solution containing purified RuBP carboxylase subjected to normal illumination (control), UV-C, UV-B or UV-A, or a total *Lemna* extract previously incubated *in vitro* for 12 h at 25 °C. Figures 4(B)–4(E) show the immunoblot analysis of the protein samples utilized in Figure 4(A), using anti-SSU (Figure 4B), anti-LSU (Figure 4C), anti-P65 (Figure 4D), or anti-(high-molecular-mass RuBP carboxylase aggregates) (Figure 4E) antibodies. These results show that the 65 kDa polypeptide, produced in either a UV-dependent or UV-independent manner, is clearly recognized by the four antibodies tested, strengthening the previous suggestion that P65 is formed by the covalent ligation of one LSU with one SSU.

Figure 4 Immunoblot analysis of *Lemna* P65 formed under a variety of conditions

Protein samples were analysed by SDS/PAGE (A) or by immunoblotting using anti-SSU (B), anti-LSU (C), anti-P65 (D) or anti-(high-molecular-mass RuBP carboxylase aggregates) (E) antibodies as described in the Materials and methods section. Lanes 1–4: *L. minor* grown under normal illumination or exposed to UV-C for 30 min, UV-B for 3 h or UV-A for 24 h, respectively; 75 μ g (A) or 22.5 μ g (B–E) of protein was loaded in each lane. Lane 5: *Lemna* crude extract incubated for 12 h at 25 °C; approx. 80 μ g (A) or 20 μ g (B–E) of protein was loaded in the gel. Lanes 6–9: a solution containing purified RuBP carboxylase was exposed, for 1 h, to normal illumination or to UV-A, UV-B or UV-C radiation, respectively; 25 μ g (A) or 6 μ g (B–E) of protein was loaded in each lane. Lane 10: purified P65 (7 μ g, A; 4 μ g, B to E).

To test if other plants, including species of economic importance, produce P65 when incubated under UV radiation, an experiment was performed in which plants of *L. minor*

(control), *Arum italicum*, cabbage, wheat, corn, pea and kidney bean were exposed for 30 min (*Lemna*) or 3 h (other plants) to normal illumination or to UV-C radiation. The plants were harvested and their soluble protein extracted and analysed by SDS/PAGE (75 μ g per lane) and by immunoblotting using anti-P65 antibodies (15 μ g per lane) (results not shown). It is clear, from the blots obtained, that all species examined produce and accumulate P65 when irradiated with UV-C. In some cases (e.g. *Lemna minor*, *Arum italicum*, cabbage and wheat) P65 is readily visible on the SDS/PAGE gel. A more detailed analysis of the SDS-gel shows the presence of a large, abundant polypeptide in corn extracts, corresponding to phosphoenolpyruvate carboxylase. It is also worthwhile to mention the lower amount of RuBP carboxylase present in corn, a C_4 plant, as well as the heterogeneity found in SSU size when the different species are compared, which is particularly evident in the case of wheat.

Characterization of P65 formation

To characterize the UV-dependent accumulation of P65 in *Lemna*, a number of experiments were performed in which compounds having known specific effects were included in the incubation medium. Thus, the incubation of *L. minor* under UV-A, UV-B or UV-C in the presence of compounds that are known to have a stabilizing and protective effect on membrane integrity and permeability, such as cholesterol (10 μ M), stigmasterol (10 μ M), β -sitosterol (10 μ M), spermidine (0.5 mM), spermine (0.5 mM) and calcium (10 mM), did not affect P65 formation (results not shown). On the other hand, the exposure to UV radiation of a solution (either in water or in 100 mM Tris/HCl buffer, pH 7.5) containing purified RuBP carboxylase (800 μ g/ml) and oxidizing agents (e.g. 10 mM oxidized glutathione), reducing agents (e.g. 10 mM DTT or 10 mM 2-mercaptoethanol), inhibitors of stress-induced oxidase systems (e.g. 2 mM KCN plus 2 mM EDTA; [46]) or superoxide dismutase (40 μ g/ml) did not prevent the accumulation of P65 (results not shown).

The observation that P65 is stable during boiling in the presence of SDS and 2-mercaptoethanol indicates that the covalent linkage between LSU and SSU does not involve disulphide bonds. Denaturation of RuBP carboxylase, by exposure of the enzyme either to acidic pH values or to high concentrations of urea or SDS, completely abolishes the formation of P65. In other words, the UV-dependent formation of P65 occurs exclusively when the enzyme is present in its native form (results not shown). This observation prompted us to incubate the activated enzyme under UV radiation, in the presence of ribulose 1,5-bisphosphate and $\text{NaH}^{14}\text{CO}_2$. At intervals, samples were collected and the polypeptides analysed by SDS/PAGE followed by autoradiography (results not shown). No radioactivity was detected in P65, suggesting that the formation of this polypeptide does not involve the carboxylation reaction. However, the rate of UV-dependent formation of P65 was altered by compounds that are known to affect the activity of the enzyme. Thus, the exposure of a solution containing purified RuBP carboxylase to UV radiation, in the presence of increasing concentrations of inhibitors of enzyme activity (fructose 1,6-bisphosphate or a mixture of the transition-state analogues 2-carboxy-D-ribitol 1,5-bisphosphate and 2-carboxy-D-arabinitol 1,5-bisphosphate) increased the rate of accumulation of P65, whereas the presence of increasing concentrations of the activator fructose 6-phosphate decreased the accumulation of P65 (Figure 5). Particularly notable was the effect of the transition-state analogues, potent inhibitors which bind tightly to the catalytic site of the enzyme [37].

Figure 5 Effects of modulators of RuBP carboxylase activity on P65 formation

RuBP carboxylase was incubated in the presence of different concentrations of 2-carboxy-D-ribitol 1,5-bisphosphate + 2-carboxy-D-arabinitol 1,5-bisphosphate (●), fructose 1,6-bisphosphate (■), or fructose 6-phosphate (▲). After 15 min, the solution containing the enzyme and the enzyme activity modulators was exposed for 1 h to UV-B radiation and analysed by SDS/PAGE to determine the amount of P65 formed (A) or assayed for RuBP carboxylase activity (B). 100% of enzyme activity corresponds to $1.6 \mu\text{mol of CO}_2 \text{ fixed} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of RuBP carboxylase.

DISCUSSION

It seems well established that exposure to UV-B radiation leads to a severe reduction in the growth and development of many plant species [10,11]. This reduction has been primarily attributed to an inhibition of photosynthesis [7], with several authors reporting a decrease in the amount and activity of RuBP carboxylase [17,18]. However, the mode of action of UV-B photoinhibition of RuBP carboxylase is not understood.

We have observed that the exposure of plant leaves to UV radiation induces the accumulation of a 65 kDa polypeptide (P65), formed by the covalent linkage of one SSU with one LSU of RuBP carboxylase. Furthermore, the rate of P65 accumulation correlates well with the rates of decrease of enzyme subunits (LSU and SSU) and with the energy of the UV radiation utilized. Prolonged exposure to UV radiation resulted in the formation of high-molecular-mass aggregates of RuBP carboxylase.

Upon exposure of a living tissue to ionizing radiation, most of the energy is absorbed by water, resulting in the production of highly reactive free radicals which can directly modify proteins, causing both inter- and intramolecular cross-links, leading to the formation of dimers, trimers or higher-order molecular aggregates [38]. In most cases, the cross-linking appears to be due to the formation of disulphide bridges, as shown by the radio-protective effect of DTT, 2-mercaptoethanol and other anti-oxidants against cross-linking, or by the observation that blockage of the exposed sulphhydryl groups by means of Ellman's reagent prevents the aggregation process.

The formation of covalent linkages between RuBP carboxylase

subunits has already been reported. Thus, intra-dimeric cross-link of LSUs of cyanobacteria and spinach RuBP carboxylases were reported by Newman and Gutteridge [39] and Ranty et al. [40]. These authors have shown that the cysteinyl sulphur atoms of Cys-247 can be selectively oxidized, with the formation of large subunits dimers (LSU_2) within the LSU_8 core of the enzyme and without the formation of higher-order aggregates. Interestingly, Ranty et al. [40] have subjected the oxidized spinach RuBP carboxylase to gel filtration under denaturing conditions (i.e. in the presence of SDS) and obtained three peaks of decreasing molecular mass (LSU_2 , LSU and SSU) in a chromatogram similar to the one illustrated in Figure 3(F). However, the disulphide bond between LSU–LSU was readily reduced with either DTT or 2-mercaptoethanol once the protein had been denatured with SDS. Other authors have reported the formation *in vivo* of higher-order aggregates of RuBP carboxylase due to disulphide cross-linking of the enzyme subunits [41–44].

The observation that P65 and the UV-dependent RuBP carboxylase aggregates are stable during boiling in the presence of SDS and 2-mercaptoethanol indicates that the covalent linkages produced do not involve disulphide bonds.

Other stress conditions have been reported to produce *in vivo* the high-molecular-mass RuBP carboxylase aggregates that are stable during boiling in the presence of 2-mercaptoethanol, such as osmotic shock, calcium starvation and, apparently, all conditions that affect membrane integrity [20,31,45,46]. We have also detected a UV-independent mode of *in vitro* P65 formation. However, formation of these conjugates appears to involve an oxidase system. The UV-dependent accumulation of P65 is not affected by inhibitors of stress-induced oxidase systems, protein synthesis inhibitors or membrane-stabilizing compounds, suggesting that the UV-dependent dimerization and polymerization of RuBP carboxylase subunits is not produced by the oxidation process reported above.

The observation that the exposure of a solution of purified RuBP carboxylase to UV radiation also induces the formation of P65 and, upon prolonged exposure, the accumulation of the high-molecular-mass conjugates further supports the view that UV radiation leads to dimerization of SSU–LSU subunits without involving other cellular components or membrane damage as intermediate steps. However, exposure of a solution containing the pure enzyme to all types of UV radiation leads to a faster response than that observed for the intact plant, probably reflecting the protective effect of many leaf components, including plant pigments. Attempts to characterize the mechanism of P65 formation or the chemical groups involved in the formation of LSU–SSU covalent cross-linkages were complicated by the observation that denaturing of the enzyme leads to a complete inhibition of P65 production by UV radiation. However, it was observed that the incubation of the native enzyme in the presence of inhibitors (such as fructose 1,6-bisphosphate or a mixture of the transition-state analogues 2-carboxy-D-ribitol 1,5-bisphosphate and 2-carboxy-D-arabinitol 1,5-bisphosphate) had a stimulatory effect on the UV-dependent formation of P65, whereas incubation in the presence of activators (such as fructose 6-phosphate) had a slight inhibitory effect. Attempts to sequence and to analyse the amino acid composition of P65, following the isolation of this polypeptide by preparative SDS/PAGE and elution from the gel, were hampered due to the great insolubility of the polypeptide. Furthermore, it is known that the determination of altered products in irradiated proteins is not a simple task, and only those products which can withstand the severe conditions of peptide bond hydrolysis will be detected [47].

The enzyme activity is strongly affected by UV radiation, the rate of decrease depending on the time of exposure and on the

energy of the radiation utilized (Figures 2C and 2F). The degree of enzyme inactivation is related to the amount of P65 accumulated, i.e. the lower the level of enzyme activity, the higher the amount of P65 formed. However, the rate of enzyme inactivation is higher than the rate of P65 accumulation (Figure 2F and Figures 1C and 1D). On the other hand, formation of P65 depends on the native state of the enzyme and is affected by the presence of enzyme inhibitors and activators. These observations suggest that P65 formation may not be directly responsible for the loss of enzyme activity, but highlight the possibility that some unknown relationship exists between conjugate formation and inactivation.

As a whole, our results indicate that the structural changes in the RuBP carboxylase molecule that lead to the covalent ligation of one LSU with one SSU of the enzyme constitute a chemical response that may contribute to the decrease in plant growth and development observed when plants are incubated under UV-B radiation. The number of plant species tested suggests that this may be a universal response, common to all photosynthetic tissues.

Lemna has been utilized as a sensitive phytoassay for the toxicity of several heavy metals [48,49]. Our results show that *Lemna minor* is a plant species that is particularly susceptible to UV radiation, especially where P65 accumulation is concerned. Its world-wide distribution as a common aquatic species makes it a valuable living system that may be used as a biological sensor in the detection of small increases in solar UV-B radiation that reach the surface of our planet due to stratospheric ozone depletion.

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