The catabolism of ribulose bisphosphate carboxylase from higher plants. A hypothesis

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Abstract

Based on current knowledge, a model is proposed for the selective catabolism of ribulose bisphosphate carboxylase (RuBP carboxylase) (EC 4.1.1.39). According to this model, the enzyme is first oxidised and subsequently covalently polymerised. However, enzymatic activities capable of oxidising the enzyme and of preferentially degrading oxidised/polymerised RuBP carboxylase in vivo have not been reported. In this work, we report the existence of both these activities and have developed methodologies capable of measuring their catalytic activities. Detection of the oxidase system involved extraction of the plant tissue incubated under conditions that are known to induce the oxidases (Lemma minor subjected to osmotic shock and, apparently, all other conditions that affect membrane integrity; Franco et al., Aust. J. Plant Physiol. 19 (1992) 297–307) and incubation under appropriate conditions with purified RuBP carboxylase as the substrate, in the presence of unknown molecular mass factor(s). Oxidation of RuBP carboxylase in vitro occurs via formation of both disulphide and non-disulphide covalent bonds between large subunits (LSUs). Detection of the oxidase system is subsequently achieved by reduced-condition sodium dodecyl sulfide-polyacrylamide gel electrophoresis (R-SDS-PAGE) (by following the accumulation of P65, an intermediate formed by non-disulphide, covalent ligation of one LSU with one small subunit (SSU)) or by anion exchange chromatography (by following the changes in the binding properties of substrate RuBP carboxylase to the fast protein liquid chromatography Mono-Q column). Detection of the proteolytic system involved the previous preparation of 3H-native, 3H-oxidised and 3H-polymerised forms of RuBP carboxylase as substrates for proteolysis. The proteolytic system exhibiting higher affinity towards oxidised and particularly polymerised RuBP carboxylase was extracted from L. minor grown under normal metabolic conditions, L. minor subjected to sulphur starvation and Triticum aestivum leaves deprived of nitrogen. Proteolysis was detected by native PAGE (by running the extract containing the proteases through a native gel containing oxidised RuBP carboxylase embedded in its matrix), R-SDS-PAGE (by following the proteolytic changes in both LSU and SSU) or liquid scintillation counting (by measuring the amount of acid-soluble radioactivity released by the action of the proteases). © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Ribulose bisphosphate carboxylase (RuBP carboxylase) (EC 4.1.1.39) catalyses the initial reactions of two important biochemical pathways, photosynthetic CO₂ fixation and photorespiration [1]. The plant enzyme is a hexadecamer composed of eight small subunits (SSU) (12–18 kDa) and eight large subunits (LSU) (51–58 kDa) [2]. Over the years, a considerable amount of information has accumulated on the synthesis of this enzyme. However, important gaps still exist in the knowledge about the degradation of RuBP carboxylase.

Under normal metabolic conditions, the pattern of RuBP carboxylase degradation appears to depend on the particular plant species [3]. Thus, it
has been reported that the enzyme suffers no degradation in *Lemna minor* and *Oryza sativa* while being synthesised [4–6]. On the other hand, the enzyme is subjected to continuous degradation at apparently different rates in wheat, maize and sorghum [3,7].

There is increasing evidence suggesting that the catabolism of RuBP carboxylase is triggered by oxidative modification of the enzyme molecule. However, several distinct types of oxidative modification have been described in the literature for RuBP carboxylase, including covalent, non-disulphide or disulphide bonds, catalysed or not by enzymes. For example, reactive oxygen species generated in illuminated or chemically treated chloroplasts from wheat have been reported to cause non-enzymatic fragmentation of LSU [8–10]. Enzymatic, non-disulphide oxidation of RuBP carboxylase has been shown to occur in *L. minor* subjected to a number of stress situations [11,12]. Non-enzymatic, non-disulphide, covalent dimerization of RuBP carboxylase subunits followed by polymerization into very large molecular mass aggregates was described in many plant species treated with UV radiation [13–15]. These non-disulphide alterations in RuBP carboxylase involve conversion of the enzyme into an oxidised and catalytically inactive form (oxidised RuBP carboxylase). Prolonged exposure to the stress leads to further oxidation and non-disulphide, covalent polymerisation of the enzyme subunits into a high molecular mass form (polymerised RuBP carboxylase). During this process, P65, an intermediate formed by the non-disulphide covalent ligation of one large subunit with one small subunit accumulates. These structural changes in the enzyme molecule are accompanied by a gradual reduction in the number of free –SH groups [11–13,16]. Disulphide cross-linking of RuBP carboxylase subunits and subsequent formation of high-order aggregates has also been reported by several authors for a number of plant species [17–20]. Other types of RuBP carboxylase oxidation involve formation of carbonyl derivatives on amino acid side chains in the enzyme from potato leaves treated with ozone [21].

The intracellular location and the proteases involved in RuBP carboxylase degradation remain to be elucidated. The two models frequently considered for the enzyme catabolism (chloroplasts versus the vacuole) are not mutually exclusive in the sense that they may complement each other or become active only under different metabolic, physiologic or pathologic conditions. Alternatively, they may both function continuously and simultaneously but, depending on the plant metabolic state, only one assumes a predominant role under a particular set of conditions. In addition, it is most conceivable that distinct proteolytic mechanisms are underway in the different stages that occur during the response of a plant to a given physiological condition. The available information suggests that, under normal metabolic conditions or during the initial, reversible stages of any other physiological condition (senescence included), the selective degradation of RuBP carboxylase may occur within the chloroplast and by the action of chloroplasts proteases. During prolonged exposure to the adverse physiological condition, when irreversible damage is imposed by the stress, the vacuolar proteolytic machinery may gradually take over, causing non-selective RuBP carboxylase degradation in a process that will lead to cell death.

Whatever the intracellular location and the proteases involved, the mechanism underlying the degradation of RuBP carboxylase and the factors that render the enzyme susceptible to proteolysis are still unknown. In this work, a methodology is presented that allowed the detection of: (i) an oxidase system capable of acting in vivo on RuBP carboxylase, and (ii) a proteolytic process that acts preferentially on the oxidised form of the enzyme.

### 2. Hypothesis

Although it seems likely that more than one pathway exists for RuBP carboxylase degradation, current knowledge suggests that, at least under some circumstances, one mechanism for the selective degradation of the enzyme involves the following sequence of events:

1. Oxidase system
2. Proteolytic system
This model is based on a number of observations. It was initially suggested by Dalling [22] that oxidative modification of RuBP carboxylase may target the enzyme for recognition by chloroplast proteases. A number of studies performed in vitro have subsequently indicated the existence of a linkage between oxidative modification of RuBP carboxylase and enhanced susceptibility to proteolytic attack by either endogenous or exogenous proteases [16,18,23]. The presence of oxidised forms of RuBP carboxylase in vivo has also been confirmed by a number of authors, either in normal metabolic conditions [18–20,26], under oxidative conditions [18], or even under physiological conditions that are not intrinsically oxidative in nature [12,20,24–26].

It remains uncertain at present whether association of oxidised (polymerised) RuBP carboxylase with membranes is an essential step preceding proteolysis. A number of studies have provided evidence that translocation of the cross-linked enzyme to membranes may be a necessary step in its degradation [13,18,20,26,27]. However, it could well be that this apparent association has no physiological significance, and merely results from aggregation of cross-linked LSU leading to co-precipitation with membranes [28].

If the proposed model is correct, then an oxidase system (capable of sequentially converting RuBP carboxylase into an oxidised and polymerised form) and a proteolytic system (exhibiting a preferential activity towards oxidised and polymerised RuBP carboxylase) must occur in plant cells under appropriate conditions. In this work, we report the existence of both these activities and develop methodologies capable of measuring their catalytic activities.

3. Materials and methods

3.1. Plant material and growth conditions

Duckweed (L. minor) was grown autotrophically under sterile conditions at 25°C, under continuous fluorescent lighting (Philips TLD 23W/33; 200 µmol quanta m⁻² s⁻¹), in the complete growth medium described previously [13]. When appropriate, the plants were incubated in complete growth medium containing 0.5 M D-mannitol (osmotic shock; [11,16]) or in growth medium deprived of calcium (calcium starvation) or sulphur (sulphur starvation; [26]).

Seeds of wheat (Triticum aestivum L.) cv Almanson were sterilised by a 2-min immersion in 70% (v/v) ethanol followed by a 20-min immersion in 1% (w/v) sodium hypochlorite. The seeds were thoroughly washed with water, imbibed in aerated water for 3 h and incubated in the dark, on wet filter paper, in Petri dishes, for 48 h at 28°C. The germinated seedlings were then grown hydroponically for periods up to 24 days under a 16 h (25°C)/8 h (22°C) light/dark cycle (fluorescent light, Philips TLD 58W/33; 200 µmol quanta m⁻² s⁻¹) in a complete growth medium containing 1.5 mM Ca(NO₃)₂, 0.5 mM MgSO₄, 0.5 mM KNO₃, 0.1 mM KH₂PO₄, 0.15 mM NaCl, 0.05 mM (NH₄)₂SO₄, 46 µM H₂BO₃, 36 µM C₆H₄FeO₇, 9 µM MnCl₂, 765 nM ZnSO₄, 197 nM NH₄VO₃, 192 nM Na₂CrO₄, 180 nM NiSO₄, 170 nM CoSO₄, and 18 nM (NH₄)₆Mo₇O₂; the pH was adjusted to 5.5. Where appropriate, 11-day-old plants were transferred and incubated for 15 days in a growth medium deprived of nitrogen. In this medium, Ca(NO₃)₂, KNO₃ and (NH₄)₂SO₄ were substituted with 1.5 mM CaCl₂ and 0.25 mM K₂SO₄, and the concentration of KH₂PO₄ was increased to 0.38 mM. The nutrient solution was changed every 4 days. The growth medium was continuously bubbled with air and the flasks topped up with new solution when required. The leaf blades of the 26-day-old wheat plants were harvested, weighed, frozen in liquid nitrogen and stored at −70°C until required.

3.2. Chemicals

L-[4,5-³H] Leucine (5.22 TBq mmol⁻¹) was obtained from Amersham International (Buckinghamshire, UK) and D-mannitol from Sigma (St. Louis, USA). The fast protein liquid chromatography (FPLC) system, Mono Q HR5/5 column, PD-10 prepacked Sephadex G-25M columns and Q-Sepharose were supplied by Pharmacia/LKB (Uppsala, Sweden). 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, USA) was used throughout.
3.3. Purification of native \(^{3}H\)-RuBP carboxylase

*Lemma* fronds were incubated in complete growth medium for 48 h in the presence of \(^{3}H\)-leucine (0.37 MBq per flask). The plants were harvested, ground with a cold mortar and pestle to a fine powder in liquid nitrogen, homogenised in 100 mM Tris–HCl buffer (pH 7.5) containing 1 mM phenylmethylsulphonyl fluoride (2.5 ml per g fresh weight), and the resulting extract filtered through gauze and clarified by centrifugation (44 000 \(x\) g, 15 min, 2\(^\circ\)C). The total soluble protein was subsequently isolated by desalting on PD-10 columns previously equilibrated with 20 mM Tris–HCl buffer (pH 7.5).

RuBP carboxylase was purified by anion-exchange chromatography. The desalted extract was loaded on the Mono Q column (5 mm \(\times\) 5 cm) or on a Q-Sepharose column (16 mm \(\times\) 12 cm) previously equilibrated with 20 mM Tris–HCl buffer (pH 7.5). The bound proteins were eluted with a continuous gradient of NaCl (0–1 M). Native \(^{3}H\)-RuBP carboxylase was eluted with 0.3 M NaCl [11].

3.4. Purification of oxidised \(^{3}H\)-RuBP carboxylase

*Lemma* fronds were incubated in complete growth medium for 48 h in the presence of \(^{3}H\)-leucine (0.37 MBq per flask). The plants were subsequently transferred to unlabelled complete growth medium containing 0.5 M d-mannitol and incubated for 36 h. The fronds were harvested and treated as described for native \(^{3}H\)-RuBP carboxylase. Oxidised \(^{3}H\)-RuBP carboxylase was eluted from the anion exchange column between 0.6 and 0.85 M NaCl [11].

3.5. Purification of polymerised \(^{3}H\)-RuBP carboxylase

*Lemma* fronds were incubated in complete growth medium for 48 h in the presence of \(^{3}H\)-leucine (0.37 MBq per flask). The plants were subsequently transferred to unlabelled completed growth medium containing 0.5 M d-mannitol and incubated for 6 days or to unlabelled growth medium lacking calcium and incubated for 14 days. The fronds were harvested and treated as described for native \(^{3}H\)-RuBP carboxylase. Polymerised \(^{3}H\)-RuBP carboxylase was eluted from the anion exchange column with 0.85 M NaCl [16,26].

3.6. Preparation of *L. minor* total cell extract for in vitro oxidation

*Lemma* fronds previously subjected to an osmotic shock (15 h) were homogenised in 100 mM Tris–HCl buffer (pH 7.5) (2.5 ml per g fresh weight). The crude extract (as opposed to the desalt extract) was utilised as the source of the oxidase system and the unidentified low molecular mass compound(s), both of which are required for the in vitro oxidation of RuBP carboxylase.

3.7. Preparation of *L. minor* and wheat total soluble protein for in vitro proteolysis

*Lemma* fronds were grown in complete growth medium or subjected to sulphur starvation for 10 days. The plants were harvested and homogenised in 100 mM Tris–HCl buffer (pH 7.5), containing 1 mM dithiothreitol (DTT) (2.5 ml per g fresh weight). The extract was clarified by centrifugation (44 000 \(x\) g, 15 min, 2\(^\circ\)C) and the supernatant (crude extract) desalted on a PD-10 column (dilution factor 1.4), previously equilibrated in 20 mM Tris–HCl buffer (pH 7.5) containing 1 mM DTT. The crude extract was diluted 1.4-fold with the same buffer. The crude and desalted extracts were subsequently used to study in vitro proteolysis.

Wheat plants were grown hydroponically in complete growth medium or growth medium without nitrogen for 15 days. The leaf blades from the 26-day-old plants were homogenised in 100 mM Tris–HCl buffer (pH 7.5), containing 1 mM DTT, 2 mM adenosine triphosphate (ATP) and 5 mM MgCl\(_2\) (2.5 ml per g fresh weight). The extract was clarified by centrifugation (44 000 \(x\) g, 15 min, 2\(^\circ\)C) and the supernatant (crude extract) desalted on a PD-10 column, previously equilibrated in 20 mM Tris–HCl buffer (pH 7.5) containing 1 mM DTT, 2 mM ATP and 5 mM MgCl\(_2\). The crude extract was diluted 1.4-fold with the same buffer. The crude and desalted extracts were subsequently used to study in vitro proteolysis.

3.8. General assays

Protein concentrations were measured by a modification of the Lowry method [29]. *Lemma* RuBP carboxylase concentrations were determined after obtaining the value of 1.54 for the extinction coefficient at 280 nm of a solution containing 1 mg
Lemna pure enzyme per millilitre. This value is in good agreement with those reported for the enzyme from tobacco (1.43) and spinach (1.64) [30,31]. Radioactivity was measured in a quench-calibrated LS3801 liquid scintillation counter (Beckman, USA). Ready-Safe (Beckman) was used as the scintillation cocktail. Samples for electrophoresis were boiled for 3 min in the presence of 2% (w/v) SDS and 0.1 M 2-mercaptoethanol and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5% (w/v) acrylamide slab gels as described previously [13]. Total polypeptides were stained with Coomassie Brilliant Blue R-250. The polypeptide bands obtained in SDS-PAGE gels corresponding to native, oxidised and polymerised RuBP carboxylase were identified as described before [13,16].

3.9. Methodology followed to detect the oxidase system

The incubation of L. minor under a variety of stress conditions (including osmotic shock, calcium starvation, darkness, CO₂ deficiency, ethanol treatment, filipin treatment and, apparently, all conditions that lead to membrane damage [12,26]) induces an oxidase system capable of catalytically inactivating RuBP carboxylase. This system may be detected and assayed in vitro using prepurified RuBP carboxylase as the substrate, in the presence of unidentified low molecular compound(s) (which may be extracted from either unstressed or stressed plants). Detection of the oxidase system may then be accomplished by SDS-PAGE (Fig. 1) or by FPLC anion-exchange chromatography (Fig. 2).

The experiment illustrated in Fig. 1 shows the SDS-PAGE analysis of the effect of the oxidase system on RuBP carboxylase. Electrophoresis performed under reducing conditions (R-SDS-PAGE, lane 1) reveals the presence of three polypeptides: the enzyme subunits LSU, SSU and P65 (a 65 kDa polypeptide formed by the non-disulphide covalent linkage between one LSU and one SSU [13]). In addition, electrophoresis performed under non-reducing conditions (NR-SDS-PAGE, lane 3) indicates formation of disulphide bonds between LSU. These results are confirmed in lane 6, where oxidised RuBP carboxylase was electrophoresed under non-reducing conditions side by side with buffer containing a reducing agent. Lateral diffusion of reducing agent during electrophoresis allows the identification of the polypeptides that contain disulphide bonds [32]. It seems clear that the oxidase system acts on RuBP carboxylase via formation of both disulphide and non-disulphide covalent bonds.

The experiment illustrated in Fig. 2 shows the effect of the oxidase system on the binding properties of RuBP carboxylase to the FPLC Mono Q anion exchanger. Native RuBP carboxylase binds to the anion exchanger at pH 7.5, being eluted with approximately 0.3 M NaCl (Fig. 2A). When acted upon by the oxidase system, the binding properties of the enzyme to the anion exchanger are predominantly modified due to an increase in its global negative charge. As a result, higher NaCl concentrations are required to elute the oxidised enzyme from the column (Fig. 2B).

3.10. Methodology followed to detect the proteolytic system that preferentially degrades oxidised RuBP carboxylase

Detection of a proteolytic system that exhibits specifically a higher activity towards the oxidised

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Fig. 1. SDS-PAGE analysis of the effect of the oxidase system on RuBP carboxylase. The pure enzyme (2 ml, 0.882 mg) was oxidised in vitro by a 1 h incubation at 4°C with 250 μl stressed Lemna crude extract. The extract was desalted and prepared for SDS-PAGE using reducing sample buffer (lane 1) or non-reducing sample buffer (lanes 3 and 6). Fifty micrograms of protein were loaded in lanes 1, 3 and 6. Reducing sample buffer was added to lanes 5 and 7. P65, LSU and SSU, 65 kDa polypeptide, large subunit and small subunit of RuBP carboxylase, respectively. Molecular masses of standards are indicated in kDa.
Fig. 2. FPLC anion-exchange chromatography analysis of the effect of the oxidase system on RuBP carboxylase. Purified RuBP carboxylase either in its native form (A) or subjected to in vitro oxidation, as explained in the caption to Fig. 1, (B) was analysed by FPLC anion-exchange chromatography on the Mono Q column previously equilibrated with 20 mM Tris–HCl buffer (pH 7.5). The bound protein was eluted with a gradient of NaCl. Peaks 1 and 2, native and oxidised RuBP carboxylase, respectively.

or polymerised forms of RuBP carboxylase requires the previous purification of radiolabelled native, oxidised and polymerised RuBP carboxylase. To this end, RuBP carboxylase was labelled in vitro by incubating, for 48 h, L. minor fronds under normal growth conditions in the presence of 3H-leucine. The fronds were subsequently harvested (for native RuBP carboxylase), or further subjected to osmotic shock during 36 h (oxidised RuBP carboxylase) or osmotic stress for 6 days, or starved for calcium during 14 days (polymerised RuBP carboxylase). Purification of RuBP carboxylase was then performed by FPLC anion-exchange chromatography as described in Section 3. These procedures allowed the efficient obtention of 3H-labelled, native, oxidised and polymerised enzyme to be used as substrates for proteolysis. The accumulation in vivo of labelled RuBP carboxylase during 48 h incubation of L. minor was possible because it is known that the Lemma enzyme is not subjected to continuous turnover under normal metabolic conditions [6]. Furthermore, the subsequent incubation of 3H-labelled Lemma fronds under conditions of osmotic shock or calcium starvation induces oxidation and polymerization of RuBP carboxylase without proteolysis of the enzyme [16, 26].

The three distinct forms of RuBP carboxylase to be used as substrates for proteolysis produce different polypeptide patterns when analysed by SDS-PAGE (Fig. 3). Native RuBP carboxylase is composed of two types of polypeptides (lane 2): LSU and SSU, with molecular masses of 52 and 14.5 kDa, respectively [6]. Oxidised RuBP carboxylase is essentially composed of three types of polypeptides (lane 3): LSU, SSU and P65, a 65 kDa polypeptide that is formed by the covalent non-disulphide ligation of one LSU with one SSU [13]. Polymerised RuBP carboxylase is composed of large molecular mass aggregates of covalently linked LSU and SSU (lane 4), covering a wide range of molecular masses, and small amounts of LSU and SSU [26].
Recent experiments performed in this laboratory have shown that the degradation of RuBP carboxylase in wheat leaves is increased by nitrogen deficiency and that the rate of enzyme catabolism is higher than that of the total soluble protein, suggesting a preferential degradation of the enzyme under these conditions [33]. Therefore, it is expected that the leaves of wheat plants deprived of nitrogen possess an enzyme system capable of degrading the oxidised form of RuBP carboxylase. To preliminary detect the presence of a proteolytic activity in nitrogen-deficient wheat leaves that acts on oxidised RuBP carboxylase, a polyacrylamide gel was prepared with pure oxidised RuBP carboxylase embedded in its matrix. A protein extract from wheat leaves deprived of nitrogen was subsequently electrophoresed, under native conditions, through the gel. When the front dye marker reached the bottom of the gel, migration was interrupted and the gel incubated at 25°C for 15 h without agitation for proteolysis to occur. The wheat proteins were then electroremoved from the gel by another 30 min of electrophoresis and the gel stained for total protein to detect the spots where the proteolysis of embedded oxidised RuBP carboxylase took place. The experiment illustrated in Fig. 4 clearly shows the presence of proteolytic activity that acts on oxidised RuBP carboxylase, in extracts prepared from wheat leaves subjected to nitrogen deficiency. A protein extract prepared from wheat leaves deprived of nitrogen was subjected to electrophoresis under non-denaturing conditions in a 5% (w/v) acrylamide slab gel containing 20 µg oxidised RuBP carboxylase per ml. When the front dye marker (m-cresol purple) reached the bottom of the gel, migration was interrupted and the gel incubated at 25°C for 15 h without agitation. The sample was subsequently electrophoresed during another 30 min and the gel stained for total protein.

Fig. 3. SDS-PAGE analysis of native RuBP carboxylase (lane 2), oxidised RuBP carboxylase (lane 3) and polymerised RuBP carboxylase (14 days calcium starvation, lane 4). Lanes 1 and 5, Molecular mass standards. a, Large molecular mass aggregates of RuBP carboxylase; P65, P65 polypeptide; LSU and SSU, large and small subunits of RuBP carboxylase, respectively. Molecular masses of standards are indicated in kDa.

Fig. 4. Detection of a proteolytic activity capable of degrading oxidised RuBP carboxylase in extracts prepared from wheat leaves subjected to nitrogen deficiency. This activity acts preferentially on P65 and LSU (Fig. 5).

The observation that wheat leaves deprived of nitrogen possess an enzymatic activity capable of degrading oxidised RuBP carboxylase does not necessarily mean that the proteolytic activity uses the oxidised form of the enzyme as a preferential substrate. In a search for endogenous proteolytic activities that act preferentially on the oxidised and polymerised forms of RuBP carboxylase, four distinct biological systems were utilised. In this context, two plant species were selected (L. minor and wheat) that are known to induce a reversible (i.e. not leading to plant death), dramatic and preferential degradation of RuBP carboxylase under suitable conditions (sulphur starvation and nitrogen deprivation, respectively) [26,33]. Extracts from these plants, either grown under normal
Solutions of native, oxidised and polymerised $^3$H-RuBP carboxylase in 20 mM Tris–HCl buffer (pH 7.5) containing 51 Bq $^3$H per ml, were prepared from $L. \text{minor}$. These samples were incubated separately at 25°C for different lengths of time (0, 0.5, 1, 2, 4 and 6 h), with desalted extracts prepared from normal (Fig. 6A) or sulphur-starved (for 10 days) (Fig. 6B) $L. \text{minor}$: 6.2 ml each extract was mixed with 6.2 ml each proteolytic substrate (native, oxidised or polymerised $^3$H-RuBP carboxylase) in the presence of 2 mM ATP and 5 mM MgCl$_2$. At intervals, aliquots were collected, the proteolytic reaction stopped and the protein precipitated with 7% (w/v) trichloroacetic acid. The aliquots were centrifuged at 10 000 × g for 10 min, the supernatants adjusted to pH 7.5 with NaOH and the precipitates dissolved in 0.4 ml of 0.2 M NaOH. The amount of $^3$H present in both supernatants and pellets was subsequently determined by liquid scintillation counting (Fig. 6). To reduce the experimental error, each value of radioactivity from supernatant or precipitate is expressed as the percentage of total radioactivity measured for that aliquot, i.e.

$$\% \text{ radioactivity in supernatant of aliquot} = \frac{\text{Bq in supernatant of aliquot}}{\text{Bq in supernatant of aliquot} + \text{Bq in precipitate of aliquot}} \times 100\%$$

We have preferred to present the data as percent values (Fig. 6) rather than the absolute amounts of radioactivity because distinct experiments corre-
spond to different values of radioactivity at time zero. Furthermore, we have selected a large number of incubation times rather than few with replicates; for this reason, no error bars are presented. The results obtained, (Fig. 6) indicate that the susceptibility of RuBP carboxylase to the action of the proteases present in *Lemna* extracts correlates positively with the degree of oxidation of the enzyme molecule, being low for native RuBP carboxylase, higher for oxidised RuBP carboxylase and even higher for the polymerised enzyme. A closer look at the data indicates that the effect of the proteases present in *Lemna* extracts does not depend on the presence of the low molecular mass cell constituents (identical results were obtained for crude and desalted extracts; data not shown). On the other hand, no difference in proteolytic activity was detected by this method when normal or sulphur-starved *Lemna* fronds were utilised as the source of proteases.

These results are consistent with the hypothesis that *Lemna* may express constitutively an enzyme system that exhibits a higher affinity towards the polymerised form of RuBP carboxylase. The absence of in vivo proteolysis of RuBP carboxylase in *Lemna* under normal metabolic conditions [6] and the large increase in enzyme degradation detected under conditions of sulphur deprivation [26] may be explained if RuBP carboxylase degradation occurs not by the induction of a protease system, but by converting the enzyme into a good substrate for the existing proteases (e.g. oxidation followed by polymerization).

To eliminate the possibility of a proteolytic activity being present in the substrate solutions, a control experiment was performed. Solutions of native, oxidised and polymerised 3H-RuBP carboxylase in 20 mM Tris–HCl buffer (pH 7.5), containing 63 Bq 3H per ml, were prepared from *L. minor*. These samples were incubated separately, at 25°C, for different lengths of time (0, 0.5, 1, 3, 6 and 12 h) in the presence of 2 mM ATP and 5 mM MgCl2. At intervals, samples were collected and the amount of radioactivity present in the acid-soluble and acid-insoluble fractions measured as already described. The results of this experiment are illustrated in Fig. 7 and show that the substrate protein solutions utilised in this work are free from contaminating proteolytic activities. This observation indicates that the proteolytic activities detected in *Lemna* (Fig. 6) or wheat (Fig. 8) extracts are indeed due to the presence of proteases in such extracts.

Fig. 7. In vitro stability of *Lemna* RuBP carboxylase to proteolysis. Native (A), oxidised (B) or polymerised (C) 3H-RuBP carboxylase solutions were incubated in buffer during the periods of time indicated. At intervals, samples were collected, the protein remaining precipitated by acid treatment, and the amount of 3H present in the supernatant (open symbols) and pellet (closed symbols) measured as explained in the text.

Solutions of native, oxidised and polymerised 3H-RuBP carboxylase in 20 mM Tris–HCl buffer (pH 7.5), containing 80 Bq 3H per ml, were prepared from *L. minor*. These samples were incubated separately, at 25°C, for different lengths of time (0, 0.08, 0.17, 0.25, 0.5, 1, 2 and 6 h), with desalted extracts prepared from normal (Fig. 8A) or nitrogen-starved (for 15 days) (Fig. 8B) wheat leaves: 8.8 ml each extract was mixed with 4.4 ml of proteolytic substrate (native, oxidised or polymerised 3H-RuBP carboxylase) in the presence of 1 mM DTT, 2 mM ATP and 5 mM MgCl2. At intervals, samples were collected and the amount of radioactivity present in the acid-soluble and acid-insoluble fractions determined as already described. The results of this experiment (Fig. 8) show that very little proteolysis occurs by the action of proteases present in the normal wheat leaf extracts. However, extracts prepared from ni-
trogen-starved wheat leaves exhibit a distinct proteolytic activity towards oxidised and polymerised RuBP carboxylase, but not towards the native enzyme. Once again, this proteolytic activity does not depend on the presence of the low molecular mass cell constituents (identical results were obtained for crude and desalted extracts; data not shown).

The results of the experiment illustrated in Fig. 8 suggest that, in contrast to *Lemna*, wheat leaves may not express constitutively, in significant amounts, a protease system specific for the oxidised form of RuBP carboxylase. Therefore, the enhanced enzyme degradation observed in nitrogen-starved leaves may result from the induction of a proteolytic system and/or the chemical conversion of RuBP carboxylase into a better substrate for proteolytic attack.

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