

2'-Carboxy-D-arabitol 1-phosphate protects ribulose 1,5-bisphosphate carboxylase/oxygenase against proteolytic breakdown

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Trypsin-catalysed cleavage of purified ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and the resultant irreversible loss of carboxylase activity were prevented by prior incubation with the naturally occurring nocturnal Rubisco inhibitor 2'-carboxy-D-arabitol 1-phosphate (CA1P), as well as with ribulose 1,5-bisphosphate (RuBP), Mg²⁺ and CO₂. CA1P also protected Rubisco from loss of activity caused by carboxypeptidase A. When similar experiments were carried out using soluble chloroplast proteases, CA1P was again able to protect Rubisco against proteolytic degradation and the consequent irreversible loss of catalytic activity. Thus, CA1P prevents the proteolytic breakdown of Rubisco by endogenous and exogenous proteases. In this way, CA1P may affect the amounts of Rubisco protein available for photosynthetic CO₂ assimilation. Rubisco turnover (in the presence of RuBP, Mg²⁺ and CO₂) may confer similar protection against proteases in the light.

Keywords: 2'-carboxy-D-arabitol 1-phosphate; photosynthesis; proteolysis; ribulose 1,5-bisphosphate carboxylase/oxygenase; stromal protease.

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39) is responsible for the assimilation of CO₂ during photosynthesis in higher plants, algae and photosynthetic bacteria. Rubisco from higher plants is composed of eight large (55-kDa) catalytic subunits and eight small (15-kDa) subunits [1]. In higher plants this enzyme is regulated by changes in pH, the concentration of Mg²⁺ and CO₂ and by other stromal activators and inhibitors, in conjunction with the light-dependent enzyme, Rubisco activase (reviewed by Parry *et al.* [2]).

Rubisco is only active when an essential lysine residue within the large subunit is carbamoylated with CO₂ followed by co-ordination of Mg²⁺ by this carbamate to form a ternary complex at the catalytic site. This process is ordered and reversible, and the equilibrium between the two forms of Rubisco (noncarbamoylated, R₀, and carbamoylated ternary complex, R) depends on the concentrations of CO₂ and Mg²⁺. 2'-Carboxy-D-arabitol 1-phosphate (CA1P) is a naturally occurring transition-state analogue of the carboxylase reaction, which has the capacity to bind tightly to the active site of Rubisco and thus inhibit both the carboxylase and oxygenase activities of the enzyme [3,4]. CA1P is found exclusively in chloroplasts [5] and is synthesized during periods of low light or darkness [6,7]. The amount of CA1P present in leaves is

dependent on the plant species and the duration of the dark period [8,9]. In *Phaseolus vulgaris*, there is enough CA1P present to block all the active sites of Rubisco while in species such as potato, soybean and tobacco there is enough CA1P present to block about 50% of the active sites of Rubisco [6,8]. On transition from dark to light, Rubisco activase promotes the release of CA1P from the catalytic site of Rubisco [10], and free CA1P is rendered noninhibitory by the action of a light-activated CA1P-phosphatase [11–13]. *In vitro*, CA1P bound to the active site of Rubisco can be released by treatment with sulfate ions and subsequently removed by gel filtration, to restore the activity of the enzyme [14].

During leaf senescence, proteins are catabolized to amino acids, which are transported to the appropriate sinks, which are frequently developing fruits [15,16]. As Rubisco is a major store of nitrogen in actively photosynthesizing mature leaves, the regulation of the breakdown of this enzyme has been subject to considerable study [17–19]. High protease activity has been detected in the vacuole [20], a ubiquitin-targeting system described in the cytosol [21] and light-dependent or ATP-dependent proteolytic breakdown described in chloroplasts [22–24]. However, there is evidence that substrates [25,26], transition-state analogues [27] and activators [24] of enzyme proteins can delay the rate of proteolytic breakdown.

X-ray-diffraction studies have shown that activation, catalysis and non-physiological inhibitors all alter the structure of Rubisco by causing the rotation of domains within the large subunit and the movement of several loop regions [28]. There is strong evidence that these structural changes can alter the susceptibility of Rubisco to proteases [29].

In this study, we have examined the effect of the naturally occurring inhibitor CA1P, ribulose 1,5-bisphosphate (RuBP) and the activation state on the breakdown of Rubisco catalysed by purified animal proteases (with a known mode of action). We have also examined the effect of CA1P on the breakdown of Rubisco catalysed by a concentrated chloroplast extract exhibiting protease activity.

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Abbreviations: CABP, 2'-carboxy-D-arabitol 1,5-bisphosphate; CA1P, 2'-carboxy-D-arabitol 1-phosphate; RuBP, ribulose 1,5-bisphosphate; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; LSU, large subunit of Rubisco.

Enzyme: Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39).

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MATERIALS AND METHODS

Materials

Rubisco was purified from wheat (*Triticum aestivum* var. *Alexandria*) and a Mediterranean shrub, *Rhamnus alaternus* [30]. Wheat Rubisco was stored in liquid N₂ and *R. alaternus* Rubisco was stored as a freeze-dried powder at 4 °C. Pancreatic trypsin, soybean trypsin inhibitor, pancreatic carboxypeptidase A and potato tuber carboxypeptidase inhibitor were all purchased from Sigma-Aldrich (Poole, Dorset, UK).

RuBP was synthesized enzymically from AMP [31]. CA1P and [2'-¹⁴C]CA1P were derived from 2'-carboxy-D-arabitol 1,5-bisphosphate (CABP) and [2'-¹⁴C]CABP, respectively, by limited treatment with potato acid phosphatase, until 50% of the organic phosphate had been released as orthophosphate [32]. CABP and [2'-¹⁴C] CABP were formed by allowing unlabelled or ¹⁴C-labelled KCN (55.1 μCi·mol⁻¹; Amersham International), respectively, to react with RuBP under weakly alkaline conditions and separating the products as described by Pierce *et al.* [33].

Activation of Rubisco

Purified Rubisco from wheat and *R. alaternus* was activated by incubation with activation buffer (100 mM bicine/KOH, 20 mM MgCl₂, 10 mM Na₂HCO₃, pH 8.2) for 40 min at 37 °C. The activated enzyme was used immediately.

Preparation of stromal extract

Stromal extracts containing chloroplast proteases were prepared from 4-week-old pea plants [34]. (Pea material was chosen as it has already been successfully used in the isolation of chloroplast proteases by others [34], while wheat chloroplast preparations have been shown to be severely contaminated with vacuolar proteases [35].) The whole procedure was conducted at 0–4 °C. A 25 g amount of fully expanded leaves was ground in a Waring blender for two 5-s periods in 150 mL ice-cold buffer containing 350 mM sorbitol, 50 mM bicine/KOH, 0.05% (w/v) BSA and 2 mM EGTA, pH 8.3. The resulting solution was filtered through two layers of Miracloth (Calbiochem) and then centrifuged for 10 min at 1500 g. The supernatant was discarded and the chloroplast pellet resuspended in 120 mL buffer consisting of 375 mM sorbitol, 35 mM bicine/KOH and 10 mM Na₂HPO₄, pH 8.3, and then centrifuged for 10 min at 1500 g. The supernatant was discarded and the chloroplast pellet resuspended in the same buffer and again centrifuged for 10 min at 1500 g. The supernatant was removed and the intact chloroplasts were broken by resuspension in 30 mL 50 mM Hepes/KOH, pH 7.7. The thylakoid membranes were then removed by centrifugation at 16 000 g for 30 min. The supernatant was concentrated in a Centriprep-30 concentrator (Amicon), until the volume was less than 1 mL, frozen in liquid nitrogen, then stored at –20 °C.

Proteolysis

Proteolysis of wheat Rubisco was performed using fixed ratios of Rubisco to trypsin and Rubisco to stromal protein extract (40 : 1 and 1 : 1, w/v, respectively). Proteolysis of *R. alaternus* Rubisco using carboxypeptidase A was at a fixed ratio of 100 : 1, by weight. Appropriate protease inhibitors were subsequently added at the same ratios, relative to Rubisco, to terminate proteolysis.

Gel electrophoresis

Wheat Rubisco was resolved into its component subunits by SDS/PAGE, using 12% (w/v) acrylamide, a proprietary gel system (Bio-Rad) and the discontinuous buffer of Laemmli [36]. Where indicated, protease digests of Rubisco were first resolved by SDS/PAGE, then immediately transblotted on to nitrocellulose paper (Hybond-C extra, Amersham International), and an epitope on the large subunit of Rubisco (LSU) visualized (unless removed by proteolysis) using a monoclonal antibody raised against wheat LSU in conjunction with a peroxidase-linked secondary antibody. Bands were subsequently visualized using an enhanced chemiluminescence kit (Amersham International).

Quantification of catalytic sites capable of binding CA1P

Activated wheat Rubisco (1 mg·mL⁻¹) was incubated with [2'-¹⁴C]CA1P (15 nmol·mL⁻¹; 6.4 μCi·μmol⁻¹) for 10 min, both before and after a 10-min exposure to trypsin (0.025 mg·mL⁻¹). Trypsin inhibitor was added to stop the action of the protease. Rubisco-bound radiolabelled CA1P was determined by liquid-scintillation spectrometry, after the removal of unbound CA1P by spin-desalting [37], using polystyrene columns (0.6 × 6.0 cm; Pierce & Warriner, Chester, UK) containing Sephadex G50 (Pharmacia) equilibrated with activation buffer.

Catalytic activity of Rubisco after exposure to proteases in the presence or absence of CA1P

Activated wheat Rubisco (1.0 mg·mL⁻¹) was incubated for 10 min, with and without CA1P (sufficient to provide a 1.1-fold

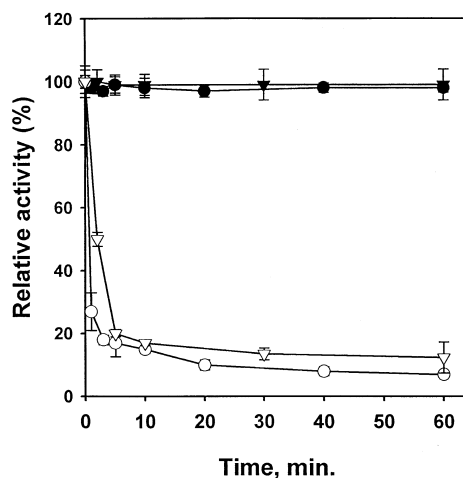


Fig. 1. Protease-dependent change in Rubisco activity in the presence and absence of specific protease inhibitors. Wheat (○, ●) or *R. alaternus* (▽, ▼) Rubisco (1 mg protein·mL⁻¹) were incubated with trypsin (○) or carboxypeptidase A (▽) 0.025 or 0.01 mg protein·mL⁻¹, respectively). In a parallel experiment, the appropriate protease inhibitor was also included, either soybean trypsin inhibitor (●) or potato carboxypeptidase inhibitor (▼), to a final concentration of 0.025 mg protein·mL⁻¹. Aliquots of 100 μL were taken at the indicated times, the digestion stopped with protease inhibitor (if not already present), and the resulting Rubisco activity determined. 100% corresponds to the activity of protease-free Rubisco, this being 379 nmol·min⁻¹·mg⁻¹ and 362 nmol·min⁻¹·mg⁻¹ for the wheat and *R. alaternus* enzymes, respectively. Values shown are the means of three replicate assays, the error bars indicating standard deviation.

excess relative to Rubisco active sites) both before and after a 10-min incubation with trypsin ($0.025 \text{ mg}\cdot\text{mL}^{-1}$). Trypsin inhibitor was added to stop the action of the protease. The resulting catalytic potential of Rubisco was measured before and after removal of bound CA1P. Such CA1P was removed by incubating the extract with 0.3 M sodium sulfate for 20 min at 25°C , followed by spin-desalting, as above, to remove the sulfate-displaced CA1P (results are given in Fig. 3).

Activated *R. alaternus* Rubisco ($1.0 \text{ mg protein}\cdot\text{mL}^{-1}$) was incubated for 10 min, with or without CA1P (1.1-fold excess), both before and after a 10-min incubation with carboxypeptidase A ($0.01 \text{ mg protein}\cdot\text{mL}^{-1}$). Digestion was terminated using potato carboxypeptidase inhibitor. Once more, the resulting catalytic potential of Rubisco was measured before and after removal of bound CA1P. Bound CA1P was released by incubating the extract with 0.3 M sodium sulfate for 20 min at 25°C , after which poly(ethylene glycol) 4000 was added to a final concentration of 25% (w/v) to precipitate the Rubisco. After 30 min at 0°C , the Rubisco precipitate was sedimented by centrifugation ($10\,000 \text{ g}$ for 10 min), the CA1P-containing supernatant discarded and the pellet resuspended in 20% (w/v) poly(ethylene glycol) in activating buffer for a further 2 min at 0°C . The Rubisco precipitate was again sedimented, as before, and finally redissolved in activation buffer, to a concentration of about $1 \text{ mg protein}\cdot\text{mL}^{-1}$ (results are given in Fig. 4).

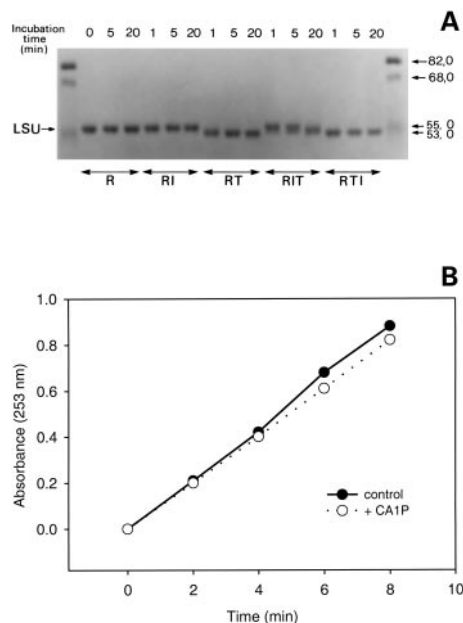


Fig. 2. Effect of CA1P on (A) the degradation of wheat LSU by trypsin and (B) the proteolytic activity of trypsin. (A) Purified wheat Rubisco ($1 \text{ mg protein}\cdot\text{mL}^{-1}$) in activation buffer was incubated at 25°C either alone (R) or after pretreatment with CA1P (RI, RIT; 1.1-fold excess relative to Rubisco active sites) in the presence (RT, RIT) or absence (R, RI) of trypsin ($0.025 \text{ mg}\cdot\text{mL}^{-1}$) for the indicated times. Digestion was subsequently stopped with soybean trypsin inhibitor ($0.025 \text{ mg}\cdot\text{mL}^{-1}$). In one experiment (RTI) trypsin treatment (for the indicated times) preceded addition of CA1P. Aliquots were taken, mixed with an equal volume of sample buffer (containing 1% SDS), boiled for 5 min, and resolved by SDS/PAGE. Each track contained $10 \mu\text{g}$ Rubisco. Protein bands were visualized by staining with Coomassie brilliant blue. (B) The trypsin catalysed hydrolysis of *N*-benzoyl-L-arginine ethyl ester (BAEE) was followed by the increase in A_{253} [51], when 1 mM BAEE in activation buffer was incubated with $2.5 \mu\text{g}\cdot\text{mL}^{-1}$ trypsin, either alone (●) or in the presence (○) of 1.6 nmol CA1P, for 10 min at 25°C .

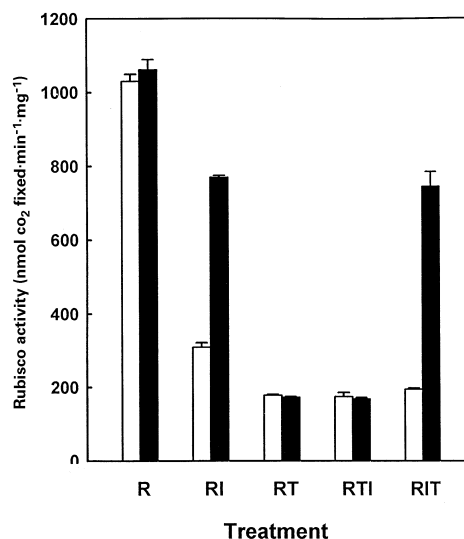


Fig. 3. Effect of CA1P on the loss of activity of wheat Rubisco caused by trypsin. Activated wheat Rubisco ($1 \text{ mg}\cdot\text{mL}^{-1}$) in activation buffer was treated as follows: R, 10 min incubation with no further additions; RI, 10 min incubation with a 1.1-fold molar excess of CA1P; RT, 10 min incubation with trypsin ($0.025 \text{ mg}\cdot\text{mL}^{-1}$); RTI, 10 min incubation with trypsin ($0.025 \text{ mg}\cdot\text{mL}^{-1}$) followed by a 10-min incubation with a 1.1-fold molar excess of CA1P; RIT, 10 min incubation with a 1.1-fold molar excess of CA1P followed by a 10-min incubation with trypsin ($0.025 \text{ mg}\cdot\text{mL}^{-1}$). Rubisco activity and protein concentration were measured after a 20-min incubation with (filled bars) or without (open bars) 0.3 M sodium sulfate, followed by spin-desalting (to remove both the sodium sulfate and the sulfate-displaced CA1P). Values shown are the means of three separate determinations with standard deviations indicated by the error bars.

Activated wheat Rubisco ($0.5 \text{ mg protein}\cdot\text{mL}^{-1}$), with or without a 10-min preincubation with CA1P (2.2-fold excess), was incubated for 24 h at 30°C in the presence or absence of an activated stromal extract ($0.5 \text{ mg protein}\cdot\text{mL}^{-1}$). The resulting catalytic potential of Rubisco was measured before and after removal of bound CA1P, as above (results are given in Fig. 5).

For all treatments, Rubisco activity was measured by incubating $20 \mu\text{L}$ sample for 2 min in $480 \mu\text{L}$ 0.2 M bicine/KOH, pH 8.2, containing 20 mM MgCl_2 , 0.4 mM RuBP and

Table 1. Amount of CA1P bound to Rubisco before and after trypsin treatment. Activated wheat Rubisco ($1 \text{ mg}\cdot\text{mL}^{-1}$) was incubated with ^{14}C -labelled CA1P (1.1-fold excess of CA1P relative to Rubisco catalytic sites) for 10 min at 25°C before or after exposure to trypsin ($0.025 \text{ mg}\cdot\text{mL}^{-1}$) for 10 min at 25°C . The amount of CA1P bound to Rubisco was determined after removal of unbound CA1P by spin-desalting. Values are the means and standard deviations of three experiments.

Sample	Rubisco-bound CA1P	
	nmol CA1P· (mg Rubisco) ⁻¹	mol CA1P· (mol Rubisco holoenzyme) ⁻¹
Rubisco + CA1P	13.5 ± 0.3	7.4 ± 0.2
Rubisco + CA1P + trypsin	10.5 ± 0.3	5.8 ± 0.2
Rubisco + trypsin + CA1P	6.2 ± 0.2	3.4 ± 0.1

Table 2. Effect of enzyme activation and substrate availability on the trypsin sensitivity of Rubisco. Wheat Rubisco ($2 \text{ mg}\cdot\text{mL}^{-1}$) was incubated for 40 min at 37°C in either 100 mM bicine/KOH, pH 8.2, or activation buffer (100 mM bicine/KOH, 20 mM MgCl_2 , 10 mM Na_2HCO_3 , pH 8.2), yielding non-carbamoylated Rubisco (R_0) and carbamoylated (activated) Rubisco (R), respectively. Both were diluted to $1 \text{ mg Rubisco}\cdot\text{mL}^{-1}$ using either bicine or activation buffer, with or without 10 mM RuBP (yielding $R_0\cdot\text{RuBP}$ and $R\cdot\text{RuBP}$) or 10 mM CA1P (yielding $R_0\cdot\text{CA1P}$ and $R\cdot\text{CA1P}$). As indicated, trypsin was added immediately ($1.67 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$) and after 10 min at 25°C , soybean trypsin inhibitor was added ($2.5 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$) to stop the digestion. In the controls, trypsin inhibitor was added before trypsin. All samples were then treated with 0.3 M sodium sulfate, followed by poly(ethylene glycol) precipitation, to remove any possible inhibitory products from the active site or in solution. The washed precipitate was resuspended in activation buffer and, after 15–20 min at 25°C , the remaining Rubisco activity (and protein concentration) was determined. The activity of the trypsin-digested samples is expressed relative to the undigested counterpart. Values are means \pm SD from three identical experiments.

Sample	Carboxylase activity [$\text{nmol}\cdot\text{min}^{-1}\cdot(\text{mg protein})^{-1}$]	Activity remaining (%)
R_0	544 ± 2	100
$R_0 + \text{trypsin}$	184 ± 1	34
$R_0\cdot\text{RuBP}$	541 ± 4	100
$R_0\cdot\text{RuBP} + \text{trypsin}$	388 ± 2	72
$R_0\cdot\text{CA1P}$	552 ± 7	100
$R_0\cdot\text{CA1P} + \text{trypsin}$	334 ± 7	61
R	840 ± 15	100
$R + \text{trypsin}$	460 ± 23	55
$R\cdot\text{RuBP}$	844 ± 40	100
$R\cdot\text{RuBP} + \text{trypsin}$	654 ± 5	78
$R\cdot\text{CA1P}$	862 ± 22	100
$R\cdot\text{CA1P} + \text{trypsin}$	742 ± 5	86

10 mM $\text{NaH}^{14}\text{CO}_3$ ($0.5 \text{ }\mu\text{Ci}\cdot\mu\text{mol}^{-1}$; ICN Pharmaceuticals) at 25°C . All assays were quenched by the addition of $200 \text{ }\mu\text{L}$ 10 M formic acid, followed by evaporation to dryness to remove all traces of acid-labile ^{14}C . The remaining ^{14}C was determined by liquid-scintillation spectrometry. In addition, the protein concentration of every sample was determined as described by Bradford [38].

RESULTS

Action of trypsin

Exposure of purified wheat Rubisco to trypsin (40 : 1 by weight) caused a loss of almost 70% of the enzyme activity within the first minute (Fig. 1, open circles). Thereafter, the decline in activity was slower, so that 5% of the activity still remained after 60 min. The loss of activity was completely prevented by the inclusion of soybean trypsin inhibitor (Fig. 1, filled circles).

During the progressive digestion of Rubisco by trypsin, aliquots were taken at the indicated times and analysed by SDS/PAGE (Fig. 2A). Trypsin was found to cause a 2-kDa reduction in the molecular mass of the LSU after only 1 min of exposure (Fig. 2A, RT) which correlates with the 70% loss of catalytic activity over the same period (Fig. 1). No effect was detected on the molecular mass of the small subunit (not shown). Incubation with CA1P before the addition of trypsin greatly reduced the rate of proteolysis, as evidenced by the

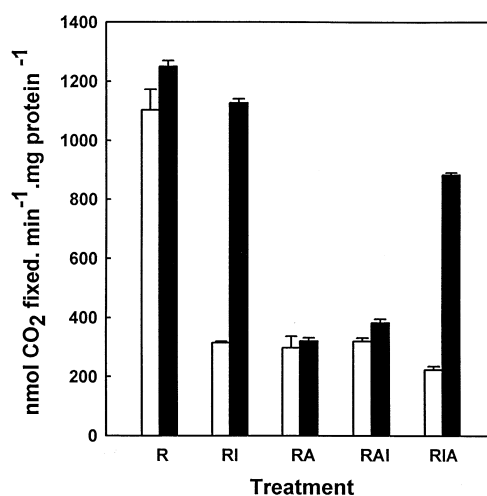


Fig. 4. Effect of CA1P on the loss of activity of *R. alaternus* Rubisco caused by carboxypeptidase A. Activated *R. alaternus* Rubisco ($1 \text{ mg}\cdot\text{mL}^{-1}$) in activation buffer was treated as follows: R, 10 min incubation with no further additions; RI, 10 min incubation with a 1.1-fold molar excess of CA1P; RA, 10 min incubation with carboxypeptidase A ($0.01 \text{ mg}\cdot\text{mL}^{-1}$); RAI, 10 min incubation with carboxypeptidase A ($0.01 \text{ mg}\cdot\text{mL}^{-1}$) followed by a 10-min incubation with a 1.1-fold molar excess of CA1P; RIA, 10 min incubation with a 1.1-fold molar excess of CA1P followed by a 10-min incubation with carboxypeptidase A ($0.01 \text{ mg}\cdot\text{mL}^{-1}$). Rubisco activity and protein concentration were measured after a 20-min incubation with (filled bars) or without (open bars) 0.3 M sodium sulfate, followed by separation of Rubisco from low-molecular-mass solutes by poly(ethylene glycol) precipitation. Values shown are the means of three separate determinations with standard deviations indicated by the error bars.

slower loss of mass by the LSU (Fig. 2A, RIT), although the removal of a short peptide of approximately 1 kDa was still visible, which progressed during the 20-min incubation period (Fig. 2A, RIT, 1–20 min). When CA1P was added after the incubation of Rubisco with trypsin, there was no amelioration of the effect of the protease, and the greater reduction in molecular mass of the LSU was again observed (Fig. 2A, RTI, 1–20 min). Furthermore, CA1P can be seen to have little (if any) direct effect on the activity of trypsin (Fig. 2B).

In the following experiments, trypsin digestion of Rubisco was allowed to proceed for 10 min, after which further proteolysis was inhibited by the addition of trypsin inhibitor. This allowed only the clearly defined changes in the molecular mass to be monitored.

The capacity of Rubisco to bind [^{14}C]CA1P is shown in Table 1. With the fully activated enzyme, it is possible to obtain a value close to the theoretical maximum of 8 mol CA1P per mol holoenzyme. After trypsin treatment, the number of molecules of CA1P that can subsequently bind to Rubisco is reduced to 3.4 per mol holoenzyme. However, preincubation of [^{14}C]CA1P and Rubisco before exposure to trypsin allows Rubisco to retain significantly more CA1P: 5.8 mol per mol holoenzyme.

The capacity of CA1P to inhibit Rubisco can be seen in Fig. 3 (compare open columns R and RI). As demonstrated by Parry *et al.* [14], the catalytic potential of CA1P-inhibited Rubisco (RI) could be measured after displacement of this CA1P with sulfate ions, followed by removal by gel filtration (Fig. 3, filled columns). Thus, the inhibitory effect of CA1P can be reversed, *in vitro* as well as *in vivo*. After a 10-min incubation with trypsin, 86% of the catalytic activity of Rubisco

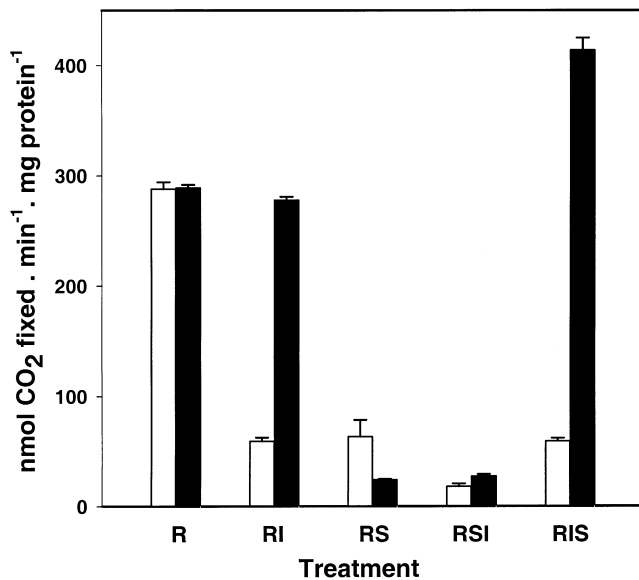


Fig. 5. Effect of CAIP on the loss of activity of wheat Rubisco caused by a pea chloroplast stromal extract. Activated wheat Rubisco ($0.5 \text{ mg}\cdot\text{mL}^{-1}$) in activation buffer was treated as follows: R, 24-h incubation with no further additions; RI, 24-h incubation alone, followed by a 10-min incubation with a 2.2-fold molar excess of CAIP; RS, 24-h incubation with activated stromal extract ($0.5 \text{ mg}\cdot\text{mL}^{-1}$); RSI, 24-h incubation with activated stromal extract ($0.5 \text{ mg}\cdot\text{mL}^{-1}$) followed by a 10-min incubation with a 2.2-fold molar excess (relative to wheat Rubisco) of CAIP; RIS, 10-min incubation with a 2.2-fold molar excess of CAIP followed by a 24-h incubation with activated stromal extract ($0.5 \text{ mg}\cdot\text{mL}^{-1}$). Rubisco activity, with (filled bars) or without (open bars) the sulfate treatment, and protein concentration were determined as described for Fig. 4. The activity of wheat Rubisco alone is shown, with the activity due to the endogenous Rubisco in the stromal extract subtracted. Values shown are the means of three separate determinations with standard deviations indicated by the error bars.

was lost (Fig. 3, RT with or without sulfate). This loss of catalytic potential was prevented if CAIP was added before (Fig. 3, RIT), but not after (Fig. 3, RTI) incubation of Rubisco with trypsin and was only apparent after sulfate treatment of the sample.

The protective effect of CAIP is likely to be caused by the structural changes to the holoenzyme which accompany binding of this inhibitor to the catalytic site. We therefore investigated whether other physiological effector molecules, which interact with the catalytic site, could alter the susceptibility of Rubisco to trypsin (Table 2). In this experiment, the ratio of Rubisco to trypsin was increased 15-fold ($600 : 1$, w/v) and the digestion allowed to proceed for 10 min. In the absence of any effectors, the activity of non-carbamoylated Rubisco was greatly reduced by trypsin digestion (Table 2, R_0) while preincubation of non-carbamoylated Rubisco with RuBP, CAIP or bicarbonate plus Mg^{2+} ions before exposure to trypsin resulted in a smaller loss of activity (Table 2, R_0 .RuBP, R_0 .CAIP and R, respectively). Even greater protection was achieved by prior incubation of Rubisco first with bicarbonate plus Mg^{2+} ions, followed by addition of either RuBP or CAIP, before exposure to trypsin (Table 2, R.RuBP and R.CAIP, respectively). In other words, Rubisco is less susceptible to the effects of trypsin when the catalytic site of the carbamoylated enzyme is occupied either by substrate or substrate analogue.

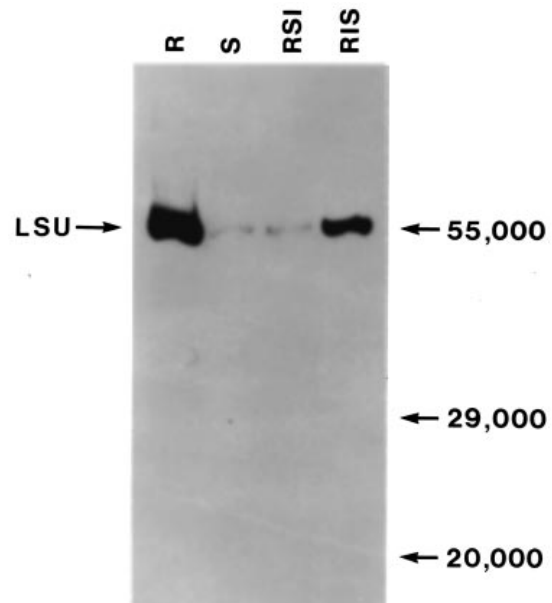


Fig. 6. Effect of CAIP on the degradation of wheat LSU by a pea chloroplast stromal extract. Activated wheat Rubisco ($0.5 \text{ mg}\cdot\text{mL}^{-1}$) in activation buffer was treated as follows: R, 10-min incubation with no further additions; S, 10-min incubation with activated stromal extract ($0.5 \text{ mg}\cdot\text{mL}^{-1}$) followed by a 10-min incubation with a 2.2-fold molar excess (relative to wheat Rubisco) of CAIP; RSI, 10-min incubation with activated stromal extract ($0.5 \text{ mg}\cdot\text{mL}^{-1}$) followed by a 10-min incubation with a 2.2-fold molar excess of CAIP followed by a 10-min incubation with activated stromal extract ($0.5 \text{ mg}\cdot\text{mL}^{-1}$). In addition (S) activated stromal extract alone ($0.5 \text{ mg}\cdot\text{mL}^{-1}$) was incubated in activation buffer for 10 min. Each sample was then resolved by SDS/PAGE, transblotted on to nitrocellulose paper, and an epitope on the LSU visualized (unless removed by proteolysis) using a monoclonal antibody raised against wheat LSU in conjunction with a peroxidase-linked secondary antibody. Each track contained $3 \mu\text{g}$ protein.

Action of carboxypeptidase A

The effects of trypsin on wheat Rubisco described above are known to be due to the removal of small peptides from the N-terminus of the large subunit [39]. To establish whether CAIP could also confer protection against C-terminal degradation of the LSU, the proteolytic inactivation of Rubisco by carboxypeptidase A ($100 : 1$, by weight) was investigated. In initial experiments, incubation of wheat Rubisco with carboxypeptidase A, for 60 min at 25°C , did not cause a reduction in the activity of the enzyme (not shown). However, carboxypeptidase A did have an effect on Rubisco purified from the Mediterranean shrub *R. alaternus* (Fig. 1, open triangles) such that about 80% of the activity was lost during a 10-min incubation with this protease at 25°C , a response that was prevented by the addition of potato carboxypeptidase inhibitor (Fig. 1, filled triangles). Using a similar approach to that described for trypsin, CAIP alone was found to inhibit *R. alaternus* Rubisco, and subsequent treatment with sulfate ions again restored catalytic activity (Fig. 4, RI). A 10-min digestion of *R. alaternus* Rubisco with carboxypeptidase A, resulted in a large fall in Rubisco activity, which was not reversed by sulfate treatment (Fig. 4, RA). The catalytic activity of Rubisco could only be restored if CAIP was incubated with *R. alaternus* Rubisco before exposure to carboxypeptidase A, and was only apparent after the sulfate-dependent removal of the same CAIP (Fig. 4, RIA).

Chloroplast proteases

In order to establish whether the capacity of CA1P to reduce the rate of Rubisco proteolysis has a physiological role, concentrated stromal extracts were prepared from pea chloroplasts by the method of Bushnell *et al.* [34] and tested for their capacity to reduce the activity of wheat Rubisco. Stromal extracts prepared from the developing leaves of 2-week-old plants exhibited little protease activity, as the activity of Rubisco was not significantly diminished in its presence, even after prolonged treatments (not shown). However, stromal extracts from fully expanded leaves of 4-week-old plants were able to reduce the activity of Rubisco, to 22% of the control value in 24 h (Fig. 5), and this inhibition was not alleviated by sulfate treatment (Fig. 5, RS). Once again, this loss of catalytic potential was prevented if CA1P was added before (Fig. 5, RIS), but not after (Fig. 5, RSI) incubation of Rubisco with the stromal extract, and was only apparent after the sulfate treatment. The stromal extracts themselves possessed Rubisco activity, which accounted for about 30% of the total Rubisco activity in the experiment described (Fig. 5). However, in a parallel experiment, this endogenous activity was quantified both before and after the stated incubation period, in the presence and absence of CA1P. In Fig. 5, the data were corrected for this endogenous activity, and so only the activity attributable to the wheat Rubisco is shown.

To confirm that the loss of Rubisco activity demonstrated in Fig. 5 was due to an endogenous chloroplast protease, the amount of Rubisco LSU remaining after exposure to the chloroplast extract was determined by Western-blot analysis (Fig. 6). In the absence of CA1P, the large subunit of Rubisco was degraded by the chloroplast extract. However, in the presence of CA1P, the LSU was still clearly visible (Fig. 6; compare lanes RSI and RIS).

DISCUSSION

Action of trypsin

The initial effects of trypsin cleavage of purified wheat Rubisco were similar to those previously reported by Gutteridge *et al.* [39], who showed that the LSU was first cleaved at Lys8 and then at Lys14, both near the N-terminus. Trypsin was thus able to liberate two peptides of ≈ 1 kDa each, and it was the removal of the second peptide that was associated with the fall in enzyme activity [39]. In the present paper, we have demonstrated that prior incubation of Rubisco with the naturally occurring inhibitor CA1P ($K_d \approx 3 \times 10^{-8}$ M) greatly reduces the rate of trypsin cleavage (Fig. 2A) and so protects Rubisco from becoming irreversibly inactivated (Fig. 3). As CA1P binding is also impaired by prior exposure to trypsin (Table 1), the loss of these N-terminal residues influences CA1P binding as well as catalysis. Conversely, the presence of CA1P (Fig. 3), RuBP, or the normal activating cofactors (Table 2) reduces the susceptibility of these residues to trypsin.

Houtz & Mulligan [40] found the same pattern of N-terminal cleavage and loss of activity when spinach Rubisco was exposed to trypsin. Furthermore, they demonstrated that this process was inhibited by incubation of Rubisco with RuBP. Similarly, Chen & Spreitzer [27] have shown that Rubisco from *Chlamydomonas reinhardtii* is protected against trypsin cleavage by the non-physiological transition-state analogue, CABP ($K_d \approx 10^{-10}$ M).

Action of carboxypeptidase A

The carboxypeptidase A-dependent release of a single amino acid residue from the C-terminus of spinach (valine) and *C. reinhardtii* (leucine) large subunits has been shown to cause a 60–65% reduction in Rubisco activity [41]. This demonstrates the importance of these C-terminal residues in maintaining catalytic activity. In the present work, the wheat enzyme was found to be resistant to attack by carboxypeptidase A. However, *R. alaternus* Rubisco was irreversibly inactivated by carboxypeptidase A (Fig. 1). We have demonstrated for the first time that this loss of activity can be prevented by prior incubation with CA1P (Fig. 4).

Thus, *in vitro*, activation, catalysis and the binding of CA1P to Rubisco confers resistance to proteolytic degradation at both the N-terminal and C-terminal regions of the large subunit. Such resistance is likely to result from conformational changes known to accompany activation, catalysis and CA1P binding. These involve movements of at least five loops and flexible elements of both the N-terminal and C-terminal regions of the large subunits [28,29]. These movements are likely to alter the accessibility of proteases to the residues that are susceptible to cleavage.

Assuming molecular masses of 550 kDa for wheat Rubisco [1], 34.6 kDa for carboxypeptidase A [42] and 23.3 kDa for trypsin [42], molar ratios of 1.7 and 6.3, respectively, can be calculated for Rubisco holoenzyme relative to these two proteases, in the experiments of Figs 3 and 4. Such high concentrations of protease relative to protein substrate are unlikely to be found *in vivo*, but serve to emphasize the potency of the protection conferred by CA1P binding on Rubisco.

Chloroplast proteases

We have examined the action of stromal proteases, to which Rubisco should be freely available. Concentrated stromal extracts isolated from mature pea leaves were able to decrease Rubisco activity (Fig. 5). CA1P has the capacity to protect Rubisco against degradation by chloroplast proteases, as it does against trypsin and carboxypeptidase A. The stromal extract was prepared from pea leaves, because intact pea chloroplasts are relatively easy to isolate and because such crude pea stromal extracts have been shown to contain at least two proteases, a serine protease and a zinc-metalloprotease, the latter with activity towards Rubisco [34].

A physiological role for protease protection by CA1P

During the day, considerable protection against proteolysis could be provided by Rubisco carbamoylation (which is promoted in the light by an ATP-dependent process catalysed by Rubisco activase) and/or by substrate turnover (Table 2). In addition, intermediates in the synthesis of CA1P [43] or tight binding inhibitors of Rubisco, distinct from CA1P, present in light-adapted leaves [14,44–46] may afford similar protection under conditions in which Rubisco activity is not saturated.

At night, the amount of RuBP in the chloroplast stroma is very low [47]. Under these conditions, in the absence of CA1P, Rubisco would become inactivated (decarbamoylated), and so become more susceptible to proteolysis (Table 2). So CA1P may ensure the continued protection of Rubisco from proteolysis at night.

As indicated in the introduction, a range of proteases are available in leaves, which would be able to digest Rubisco, particularly after the onset of senescence. In a recent study by He *et al.* [48] Rubisco activase deficient tobacco plants were

found to contain unusually large amounts of less active Rubisco during senescence. This situation could have arisen if the Rubisco in such activase-deficient plants had sequestered protective tight-binding inhibitors, the subsequent removal of which was impaired (owing to the lack of activase).

Relatively few plant species contain sufficient CAIP to protect every available Rubisco catalytic site [6,49]. Only in the case of such abundantly supplied species could CAIP afford complete nocturnal protection. However, as shown by the differential sensitivity of Rubisco from different plant species to carboxypeptidase A (above), Rubisco from different species may not be equally susceptible to degradation by the available proteases. A consequence of this may be the variable rates of Rubisco breakdown seen in different species [50] and may explain why different species contain different amounts of CAIP at night.

Thus, while the sequence of events and the order of proteolytic attack during Rubisco degradation *in vivo* is not clear, CAIP may be an important factor in the process. Further studies will provide more information on the physiological conditions under which this protective system operates.

We are acquiring new data which show that naturally occurring Rubisco inhibitors, present in illuminated leaves [14,44,45], may protect Rubisco from stress-induced inactivation ([52] and S. Khan, P. J. Andralojc & M. A. J. Parry, unpublished data). Hence, the protection conferred on Rubisco by CAIP may be one aspect of a more general type of protective strategy *in vivo*.

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