Inactivation of ribulose-bisphosphate carboxylase by limited proteolysis

Loss of the catalytic activity without disruption of bisphosphate binding or carbamylation

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Limited proteolysis of ribulose-bisphosphate carboxylase with trypsin or endopeptidase Lys C causes the loss of the carboxylase and oxygenase activities, without disrupting the binding of bisphosphates or the reactions normally associated with activation of the enzyme. Gel electrophoresis of the non-denatured enzyme indicates that the L_8S_8 quaternary structure of large and small subunits is unaffected by this treatment. However, electrophoresis in denaturing conditions shows that the L subunit is smaller as a result of the removal of two short polypeptides, each approx. 1000 Da. The loss of activity is associated with the removal of the second peptide. The amino acid composition of the isolated peptides indicates that both originate from the N-terminus of the L subunit. Over protracted periods of exposure to the proteases a third smaller peptide is also released from the N-terminus of the L subunit. The S subunit is not affected by these treatments. The carboxylase isolated from a number of photosynthetic species exhibits a differential response to this limited proteolysis.

Ribulose-bisphosphate carboxylase *Enzyme inactivation* *Proteolysis* *Bisphosphate binding* *Carbamylation*

1. INTRODUCTION

Ribulose-bisphosphate (P_2) carboxylase, the most important CO_2-fixing enzyme of photosynthetic organisms, catalyses a competing oxygenase reaction [1,2]. The opportunity now exists of introducing specific structural changes in the enzyme [3], to attempt to engineer either a better carboxylase or to reduce the wasteful oxygenase activity. It is helpful when designing these modifications rationally to know at least all of the regions of the protein sequence, if not the individual amino acids, that contribute to the active-site structure.

In the absence of X-ray crystallographic information, the use of typical, e.g. active-site affinity reagents [4] and atypical, such as diazomethane [5], reactions of protein chemistry in addition to sequence comparisons of the enzyme from many diverse organisms has pinpointed at least 6 conserved regions of the large (L) subunit that must be close to the catalytic site. One particular region from residue 195 to 204 in the spinach enzyme contains two conserved amino acids of known function; lysine at position 201 is the residue that is carbamylated by CO_2 during activation [5], and aspartate at position 198 is involved in the coordination of the essential metal ion [3].

Apart from some photosynthetic bacteria, the usual quaternary structure of the enzyme is a complex hexadecameric arrangement of L and small (S) subunits. The carboxylase from some photosynthetic organisms, e.g. cyanobacteria, readily loses the S subunit from the L_8S_8 structure,

Abbreviation: APMSF, *p*-amidinophenylmethylsulphonyl fluoride

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generating an inactive L_8 core. Activity can be readily restored on readdition of the S subunit [6]. Although catalysis is lost as the S subunit is removed, the protein is still able to bind bisphosphates effectively [7] and can be carbamylated. Thus, either the S subunit contributes amino acids essential for catalysis, or it induces changes to the conformation of the L subunit to bring all essential amino acids into the correct orientation to restore activity.

Here, we present data that show that the carboxylase from higher plants, exposed to limited proteolysis, loses catalytic activity without any significant disruption of the bisphosphate-binding site or the reactions associated with activation. However, unlike the enzyme from cyanobacteria, it still retains the L_8S_8 quaternary structure, although the L subunit is reduced in size.

2. MATERIALS AND METHODS

Ribulose-P_2 carboxylase was purified from wheat, spinach and barley as described [8,9]. The carboxylase from *Rhodospirillum rubrum* was the recombinant enzyme expressed in an *E. coli* host as described by Gutteridge et al. [3] (see also [10]).

2.1. *Activation and assay*

The purified enzyme from the higher plant sources was activated by dissolving the protein (stored as a freeze-dried powder) in 0.1 M Bicine (pH 8.2) containing 10 mM $NaHCO_3$ and 40 mM $MgCl_2$ and incubated for 30 min at 40°C to obtain the maximum activity. The *R. rubrum* carboxylase was activated in the same buffer with 50 mM $NaHCO_3$ and 40 mM $MgCl_2$ for 30 min at 25°C. The activated enzymes were then stored for the duration of the experiments at 25°C. The carboxylase activity was determined as the incorporation of $^{14}CO_2$ into acid-stable products after ribulose-P_2 carboxylation [11,12].

2.2. *Proteolysis*

Proteolysis of the carboxylase species was performed using a fixed ratio of trypsin (TPCK-treated from Sigma) or endopeptidase Lys C (Boehringer) to enzyme as described in section 3. The carboxylase concentration was usually 15 mg/ml. For the preparation of peptides, proteolysis was usually performed in NH_4HCO_3 (10 mM) pH 7.5. The carboxylase was freed of non-volatile salts by rapid centrifugal gel filtration through Sephadex G-25 (fine) [13] into 10 mM NH_4HCO_3.

The peptides produced during trypsinolysis were isolated either by centrifugation through semi-permeable cones (Amicon CF50A), dialysis against a large volume of the volatile buffer, or gel filtration using a 50 × 2.5 cm Sephadex G-25 (fine) column. Centrifugation with the cones was adopted to follow the time course of peptide release with either protease and 0.01 ml APMSF (1 mM) was added to the centrifuge tubes to inhibit any small amount of protease that passed through the membrane with the peptides. The cones were spun at 1000 × *g* for 5 min. Separation of individual peptides was achieved using 10 × 10 cm TLC silica gel plates (Whatman) developed in a butanol, acetic acid, water (3:1:1) solvent.

The amino acid composition of the peptides, either as mixtures or isolated from the TLC plates, was determined by automated amino acid analysis (LKB 4400) after acid hydrolysis in 6 N HCl at 105°C for 22 h in sealed evacuated vials.

The inactive enzyme resulting from trypsinolysis was also recovered free of the protease and peptides by passage through a Sephacryl S200 column (2 × 40 cm) equilibrated with 10 mM NH_4HCO_3.

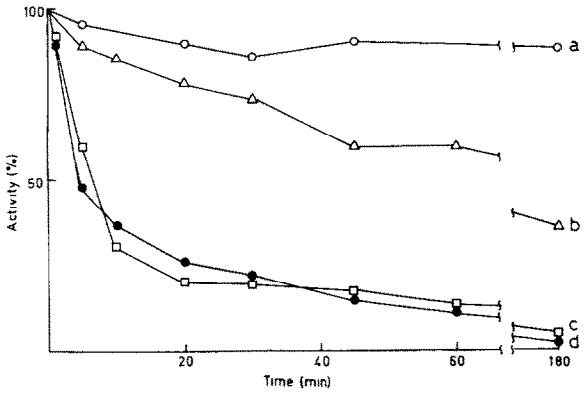

Fig.1. Inactivation of ribulose-P_2 carboxylase from different photosynthetic species. In all cases, 15 mg/ml of the carboxylase in 10 mM NH_4HCO_3 (pH 7.5) was treated with trypsin (0.04 mg/ml) at 25°C and aliquots taken at set times for assay. The loss of the carboxylase activity was determined for the enzymes from (a) *R. rubrum*, (b) barley, (c) wheat and (d) spinach.

2.3. *Gel electrophoresis*

Electrophoresis in polyacrylamide gels was performed using a slab apparatus (Biorad) and the discontinuous buffer system of Laemmli [23]. Gel and reservoir buffers were supplemented with 0.1% (w/v) SDS for the electrophoresis of denatured proteins. Gels were stained as in [8].

3. RESULTS

The loss of activity of the carboxylase from wheat which occurs with the addition of a small quantity of trypsin is shown in fig.1. With a protease/carboxylase ratio of 1:400 (w/w), 80% of the activity was lost in 20–30 min. The rate of inactivation was not constant, being initially fast, but then proceeded for a further 2 h before depleting the remaining 20%. Fig.1 also compares the effect of the same concentration of trypsin on other species of the carboxylase. The purified enzyme from spinach was inactivated as rapidly as the wheat enzyme, whereas the carboxylase from barley was much more resistant to the treatment. The enzyme from the photosynthetic bacterium *R. rubrum*, which is composed of a dimer of L subunits and has no S subunit, was only marginally affected by the trypsin.

Clearly, the susceptibility of the carboxylase to the proteolysis could simply be due to a general effect of the trypsin cleaving randomly at basic residues of either the L or S subunits. Alternatively, the process might be much more specific, particularly as lysine residues have been identified as being essential for catalysis. Two of these basic amino acids occur at positions 175 [14] and 201 [15] of the primary sequence, that along with Lys

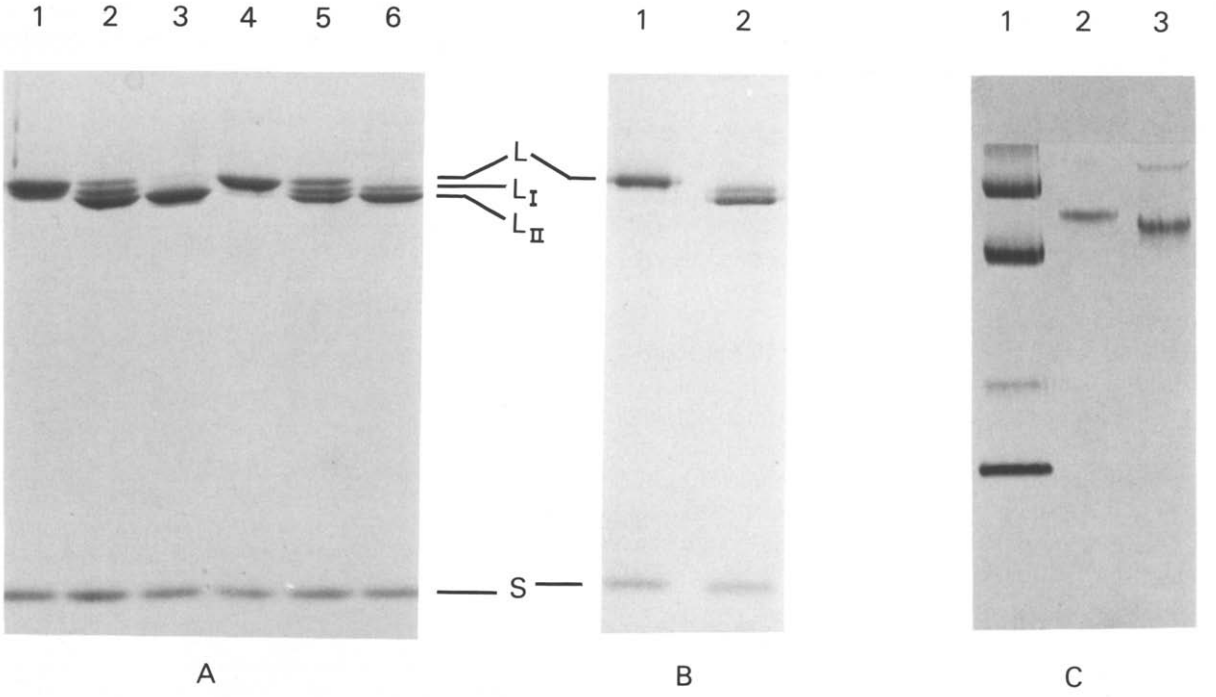

Fig.2. Polyacrylamide gradient gels of trypsinolysed wheat carboxylase. (A) A gradient gel (10–20%), containing 0.1% SDS, of the enzyme at different times after exposure to trypsin showing that the site of action of the protease is the L subunit. Tracks 1–3, carboxylase samples taken at 10 s, 8 and 30 min after proteolysis with 40 μg/ml trypsin. Tracks 5 and 6, carboxylase treated for 15 and 45 min with 8 μg/ml trypsin. The carboxylase concentration was 15 mg/ml and the proteolysis was halted by injecting samples into boiling buffer containing 0.1% SDS. Track 4, untreated carboxylase. (B) A similar gel of the inactive carboxylase, freed of the protease after 45 min before electrophoresis, compared to the untreated enzyme (left track). (C) The carboxylase was the same as that in (B) except that the proteolysis was for 75 min and the electrophoresis on a 4–20% gradient non-denaturing gel. The standard proteins from the top in the left-most track were thyroglobulin, ferritin and BSA.

334 [15] compose part of the active site. Similarly, arginine groups have also been implicated, possibly binding the phosphate groups of the substrate ribulose-P_2 [16] or bisphosphate effectors.

To locate the target of the trypsin action, untreated and trypsinolysed wheat carboxylase were electrophoresed on an SDS-polyacrylamide gradient gel. Fig.2A is a photograph of the stained gel which shows that the cleavage is far from random. The S subunit was unaffected by trypsin whereas the L subunit lost two peptides, I and II, peptide I being apparently greater in size than II.

Tracks 5 and 6 of fig.2A show the effect of exposing wheat carboxylase to the same concentration of trypsin used to produce the inactivation data plotted in fig.1c. The L subunit is significantly degraded to two lower M_r polypeptides L_I and L_{II} after 10 s. The proteolysis was repeated with 5-fold less trypsin to relate more closely the loss of activity with the release of either one or both peptides (fig.2A, tracks 1–3). In track 2 the distribution of the L subunit after 15 min proteolysis is approx. 30% intact, 70% without peptide I (i.e. $L_I + L_{II}$) and 40% without II. The activity of the enzyme determined at the same time as the sample was denatured for electrophoresis was 40% of an untreated carboxylase control. Thus the loss of peptide II must account for the major part of the inactivation (see section 4).

The effect of trypsin on the activity of the carboxylase could be due to a number of factors, especially as catalysis is dependent on, e.g. activation reactions such as carbamylation [17] and an intact quaternary structure. Any change to the gross structure of L and S subunits was investigated by electrophoresis in non-denaturing conditions. Enzyme that had been treated with trypsin was first freed of the protease by gel filtration (see section 2). The mobility of the native carboxylase was compared with that of the treated enzyme by electrophoresis on a gradient gel (fig.2C) to show that the L_8S_8 quaternary structure was not dramatically affected. Confirmation that the trypsinolysed and protease-free carboxylase was composed of S subunits associated with the modified L subunit was obtained by running an SDS gel of the treated enzyme (fig.2B). In this particular example, trypsinolysis was not complete before gel filtration and the L subunit therefore ran as a doublet of L_I and L_{II}.

There are also direct ways of investigating disruption that may have occurred to the active site. The activation process, which is a necessary pre-requisite before catalysis can proceed, involves the carbamylation of Lys 201 at the active site by CO_2 and then co-ordination of the essential metal ion [17], usually Mg^{2+}. The activating CO_2 and the metal can be trapped irreversibly with the tight binding transition state analogue, 2-carboxyarabinitol bisphosphate (2CABP) [18]. When the trypsinolysed carboxylase (protease-free) was carbamylated with $H^{14}CO_3^-$ in the presence of Mg^{2+} and 2CABP, 75% of the enzyme molecules had bound $^{14}CO_2$. This compared favourably with the untreated enzyme (80%). Furthermore, the rate of release of the bound $^{14}CO_2$ from the quaternary complex was equally slow for both forms of the carboxylase.

The gel data of fig.2 indicate the loss of 2 peptides from the L subunit. Indeed, 2 peptides

Table 1

The amino acid compositions of the peptides released from wheat ribulose-P_2 carboxylase

	N-terminal sequence	Peptide I	Peptide II	C-terminal sequence
D	–	tr.	tr.	2
T	2	1.6	–	1
S	1	0.7	–	–
E	2	1.3	–	2
P	1	0.9	–	1
G	2	–	1.8	–
A	1	–	1.2	1
V	1	–	0.8	1
I	–	–	–	2
Y	–	–	tr.	–
F	1	–	1.0	2
K	2	1.0	1.2	2

The compositions of peptides I and II were determined from acid hydrolysate of mixtures released by either trypsin or Lys C or isolated from thin-layer plates. The amino acids were quantitated with respect to a norleucine internal standard and then normalised relative to lysine for peptide I and phenylalanine for peptide II. The ratio of I to II was constant as the quantities increased during the proteolysis (see section 3). The N- and C-terminus compositions were determined from DNA sequences. tr., trace

predominated and could be isolated after trypsinolysis of the carboxylase. The compositions of the peptides cleaved from the L subunit of the wheat enzyme were determined, being collected and isolated by various methods (see section 2) and analysed for their amino acid content after acid hydrolysis. It is clear from a comparison of these compositions shown in table 1, with the N- and C-terminal sequences deduced from the DNA sequence for wheat L subunit (T. Dyer and M.W. Saul, unpublished), that both peptides originate from the N-terminus.

The mixture of the two peptides obtained after trypsinolysis contained variable amounts of some other amino acids possibly as a result of the accumulation of other peptides from the carboxylase. Thus, to ensure that the effect of proteolysis on the carboxylase activity and structure could only be due to the loss of N-terminal peptides, a second protease with greater specificity, endopeptidase Lys C, was used. Lys C produced identical results to trypsin at similar concentrations, except the peptide mixtures were significantly less contaminated with other amino acids.

4. DISCUSSION

The only example of the inactivation of ribulose-P_2 carboxylase without the loss of other functions such as substrate binding or activation is the enzyme from cyanobacterial sources that readily and reversibly loses the S subunit [6]. It was considered, therefore, that limited trypsinolysis of the higher plant carboxylase might be disrupting the interaction between the L and S subunits. However, there is no evidence from the gel electrophoresis that the S subunit dissociates from the intact enzyme, or indeed that the interaction with the L subunit is weakened (not shown), rather it is the removal of a short length of the L subunit that destroys the activity.

This is the first report that the region encompassing the N-terminus of the enzyme is important for catalysis. From the time course of peptide release revealed by SDS gels, at the highest concentrations of protease the degradation of the carboxylase is apparently complete within 70 min, although some 5–10% of the activity still remains. Thus, the peptides that are removed may not contribute amino acids essential for the reaction mechanism, but most likely ensure the correct geometry of the active site for catalysis to proceed. The absence of any disruption to the amino acids involved in activation, i.e. carbamylation, metal co-ordination or even to bisphosphate binding, suggests that additional residues are utilised for these processes. For example, amino acids that catalyse proton abstraction from or donation to ribulose-P_2 [19] which are essential to the reaction mechanism of the carboxylase [20] could be displaced without disturbing activation or substrate binding. However, the susceptibility of this region of the L subunit to the proteases indicates that it must be exposed to the solution on the surface of the polypeptide and not buried between either two L subunits or an S and L subunit in the hexadecameric structure of the intact enzyme.

The particular lysine in the N-terminal sequence (see fig.3) that is the first site of proteolytic action cannot be resolved unambiguously. The amino acid compositions of isolated peptides indicate that cleavage at the lysine at position 8 generates L_I and peptide I and the remaining L subunit is then further processed at position 14 to give L_{II} and peptide II. However, it is also likely that some L subunit molecules are cut directly at position 14 generating a peptide composed of I and II that may only then be cleaved by trypsin or Lys C. Thus, there is apparently no obligatory process of $L–L_I–L_{II}$, although over the shortest periods of exposure to the proteases the gel data of fig.2 suggest there may be a slight preference for Lys 8. Only peptide II from the wheat enzyme has yielded to sequencing confirming its location as 9–14 of the L subunit. Peptides I or I + II do not sequence because of a blocked N-terminus. The L subunit of

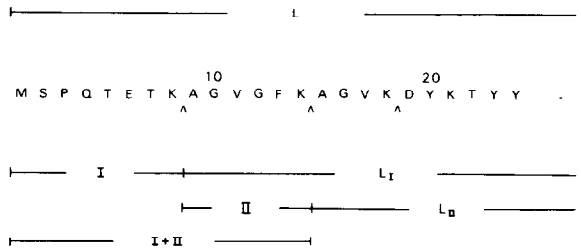

Fig.3. The N-terminal sequence of wheat carboxylase (T. Dyer and M.W. Saul, unpublished) showing the proposed position of action of trypsin.

wheat carboxylase is further degraded, but only after protracted exposure to the proteases. The presence of increasing amounts of valine, glycine, alanine and lysine in acid hydrolysates of the isolated peptides suggests that Lys 18 is the next most susceptible position on the L subunit.

The other species of carboxylase inactivated by trypsin produced similar results to the wheat enzyme concerning the pattern of L subunit proteolysis. Certainly, the peptides released from spinach carboxylase give the expected composition of that N-terminal sequence. The rates of peptide release also correlated well with rates of inactivation of the various carboxylases. The reason for these differential rates obtained with, e.g. barley compared to wheat, is readily explained by the replacement of Lys 14 by a glutamic acid. However, the carboxylase from tobacco which has about the same rate of inactivation as barley does not have this difference. Presumably, other regions of the tertiary or quaternary structure of these enzymes determine the resistance to proteolysis.

An important aspect arising from the amino acid composition of the Lys peptides isolated from spinach or wheat carboxylases is that the L subunit is apparently not post-translationally modified, i.e. the N-terminal composition is that expected from the DNA sequence. However, the ease with which trypsin can remove the N-terminal peptide suggests that in those reports of L subunit processing, e.g. in the enzyme from barley [21] (but see also [25]) and spinach [22], it may have been due to general proteolysis rather than a specific modification post-translationally, a process that would have been readily detected as a fall in the specific activity of the enzymes being investigated. Clearly, the susceptibility of the carboxylase to protease action may explain the variability of the specific activity of the enzyme that often accompanies its isolation and purification (see e.g. [24]).

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