S16-012

A mutation in the large subunit of tobacco Rubisco reduces the decline in activity during catalysis

FG Pearce, TJ Andrews, HJ Kane, RE Sharwood & SM Whitney.

Research School of Biological Sciences, Australian National University, P.O. Box 475, Canberra, ACT 2601, Australia, e-mail gpearce@rsbs.anu.edu.au, Fax 61-2-6125-5075

Keywords: *Rubisco, fallover, Rubisco activase, diketone, mutagenesis*

Introduction

The gene encoding the Rubisco large subunit of tobacco has been mutated specifically at codon 335 using plastid transformation, converting it to encode valine instead of leucine (Whitney *et al*. 1999). Leu-335 is located on loop 6 of the large subunit, adjacent to the catalytically important Lys-334 residue. The Leu-335 residue also makes van der Waals contacts with the P2 phosphate of the substrate.

As a result of this substitution, the mutant enzyme's maximal carboxylation rate and specificity for CO_2 were reduced to less than 25% of the wild-type values. The Michaelis constants for CO_2, O_2 and D-ribulose-1,5-bisphosphate (RuBP) were also reduced. Further studies have now been carried out to determine the effect of the mutation on other activities in the active site.

Materials and Methods

Wild-type (Leu-335) and mutant (Val-335) tobacco Rubiscos were purified by crystallisation (Servaites, 1985) and their concentration measured by absorbance at 280 nm using a molar extinction coefficient of 0.7. A recombinant Rubisco activase gene from tobacco with a hepta-His tag fused to the C terminus (Whitney and Andrews, unpublished) was expressed in *E. coli*. The His-tagged Rubisco activase was purified using NiNTA agarose (Qiagen). [^{3}H]-labelled and unlabelled RuBP were synthesised as described (Kane *et al*. 1998).

Rubisco activity was measured at 25ºC using a spectrophotometric assay (Lilley & Walker, 1974). Exponential curves were fitted to calculate activity rates and rate constants (Edmondson *et al*. 1990). For the fallover assays, activated Rubisco (0.2 – 0.6 μg ml^{-1}) was used to initiate the reactions and Rubisco activase (20 μg ml^{-1}) was added 900 or 1400 seconds later. For assays used to measure the activation of RuBP-inhibited Rubisco (ER) by Rubisco activase, ER was formed by incubating 200 μg of non-carbamylated Rubisco (E) with 0.5 mM RuBP (R) for 30 min before adding to reactions (10 μg ml^{-1} final concentration) containing Rubisco activase (40 μg ml^{-1}) (as described by Lan & Mott (1990)).

Ligand binding was tested by activating Rubisco (400 μg ml^{-1}) in buffer (50 mM Tricine-KOH, pH 8, 15 mM $MgCl_2$, 15 mM $NaHCO_3$) and then incubating with 40 μM [^{14}C]-CPBP (a mixture of D-2'-carboxyarabinitol-1,5-bisphosphate (CABP) and D-2'-carboxyribitol-1,5-bisphosphate). [^{14}C]-CABP bound to Rubisco (E-CABP) was separated from unbound [^{14}C]-CPBP by gel filtration using Sephadex G-50 (1 x 20 cm) equilibrated with buffer. [^{12}C]-CPBP (300 μM) was then added to the E-[^{14}C]-

CABP fraction and incubated a further 9 days at 25ºC before re-chromotographing. The amount of radioactivity in the fractions was measured by scintillation counting.

To test the binding of RuBP derivatives to activated Rubisco, [^{3}H]-RuBP was incubated at 25ºC for 3 h with 1 mM $CuSO_4$ as described (Kane *et al*. 1998). The Cu^{2+} oxidised RuBP (8 mM) was incubated with 650 μg ml^{-1} activated Rubisco at 25ºC for 30 min before concentrating the sample with an Ultrafree-MC membrane (Millipore) by centrifugation. Samples were washed with four volumes of buffer (50 mM EPPS-NaOH, pH 8, 15 mM $MgCl_2$, 15 mM $NaHCO_3$, 1mM EDTA) before the Rubisco in the retentate was denatured with SDS (1% w/v). After centrifuging again, the filtrate was loaded onto a Mono-Q 5/5 column equilibrated with 10 mM EPPS-Borate, pH 8.0, and the RuBP derivatives were separated using a NaCl gradient. The radioactively labelled compounds were detected by online scintillation counting.

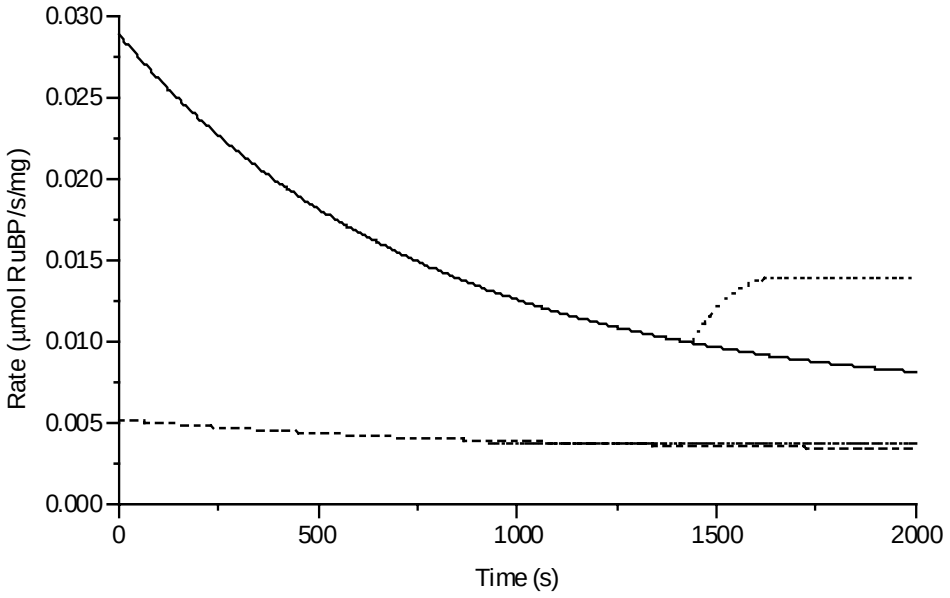


Figure 1: The decline in Rubisco activity over time during assay *in vivo*. Leu-335 is shown without activase (——) and with Rubisco activase added after 1400 s (·····). Val-335 is also shown without Rubisco activase (– – –) and with Rubisco activase added after 900 s (– · – · –).

Results and Discussion

Val-335 Rubisco displays reduced fallover

When Rubisco is assayed *in vitro*, its activity declines progressively. This phenomenon is termed 'fallover'. It results from the gradual accumulation of inhibitory compounds at the active sites. These inhibitors may be formed by the enzyme in 'misfire' reactions, or be present at low concentrations as contaminants in RuBP preparations (Kane *et al.* 1998).

Activity of the Leu-335 form of Rubisco declines to a final rate that is less than 30% of the original rate, with half the decline occurring in the first 7 minutes (Figure

1). In contrast, the Val-335 Rubisco activity declines to only 60% of the original rate, and takes over 10 minutes for half the decline to happen.

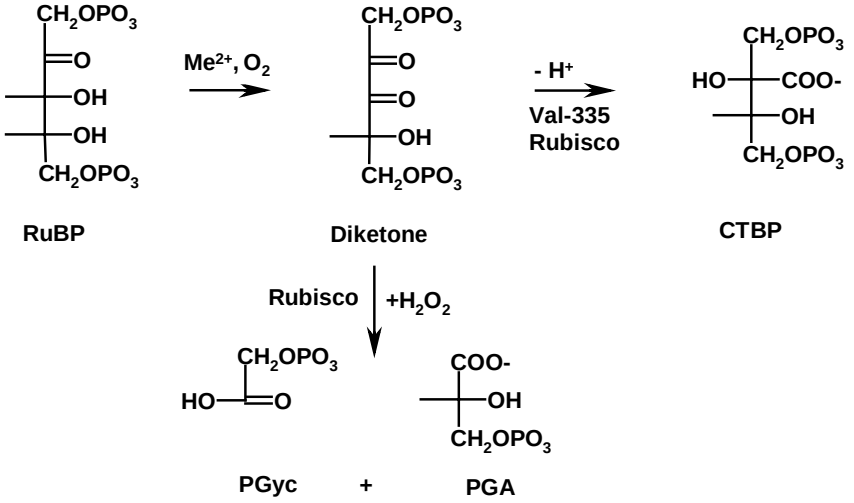
If this decline in activity occurred *in vivo*, Rubisco would be less effective. To prevent this happening, plants contain the enzyme Rubisco activase, which uses the energy from ATP hydrolysis to loosen the active site of Rubisco and release the ligand. Addition of Rubisco activase enzyme to Leu-335 Rubisco partially restores activity and linearises the rate while addition of Rubisco activase to the Val-335 form does not have as great of an effect (Figure 1).

Val-335 Rubisco is able to bind ligands

Is Val-335 Rubisco resistant to fallover inhibition because it binds the inhibitory compounds less tightly? If the mutated loop 6 closes less precisely over the active site, the ligands might bind less tightly. This was tested by incubating activated enzyme with [^{14}C]-CABP, a tight-binding inhibitor which is a known mimic of a reaction intermediate. Mutant and wild-type forms released similarly small amounts of the inhibitor over 9 days, >80% of the CABP remaining bound in both cases. This indicates that the substitution of the residue does not loosen the binding of inhibitors.

Rubisco activase still removes bound ligands

Rubisco requires carbamylation of a lysine residue and the subsequent binding of a Mg^{2+} ion to become catalytically active. RuBP can bind to the uncarbamylated active site to form an inactive complex from which it escapes very slowly without the assistance of Rubisco activase. Incubation of the Rubisco:RuBP complex with activase showed that activase was similarly effective with both forms of Rubisco in releasing the RuBP and allowing activation to occur (data not shown). Therefore, the mutation does not appear to impair the ability of Rubisco activase to release inhibitors from the active site.



Scheme 1. Formation of the diketone inhibitor by oxidation of RuBP, and subsequent peroxidative cleavage by Rubisco to form PGyc and PGA, or rearrangement to CTBP by Val-335 Rubisco**.**

Val-335 Rubisco converts the diketone to CTBP

The diketone D-pentodiulose-1,5-bisphosphate is thought to be one of the inhibitory compounds that causes fallover (Kane *et al.* 1998). It is produced by the oxidation of

RuBP, such as in the presence of Cu^{2+} ions (Figure 2A and Scheme 1). It could bind to both the Leu-335 and Val-335 forms. The Leu-335 Rubisco had less inhibitors bound than the Val-335 Rubisco (Figure 2). The Val-335 Rubisco was also able to catalyse the conversion of the diketone to CTBP (2'-carboxytetritol-1,4-bisphosphate).

In the presence of trace amounts of H_2O_2, Rubisco is able to convert the diketone into 2-phosphoglycolate (PGyc) and 3-phosphoglycerate (PGA), which are subsequently released (Scheme 1). The Leu-335 Rubisco may have less bound inhibitor due to the release of the diketone in this manner. However the Val-335

Rubisco is able to catalyse the rearrangement of the diketone to CTBP (Scheme 1), which remains bound to the enzyme. The Lys-334 → Ala mutant of *Rhodospirillum rubrum* also rearranges the diketone to CTBP which, in that case, readily dissociated (Harpel *et al.* 1995).

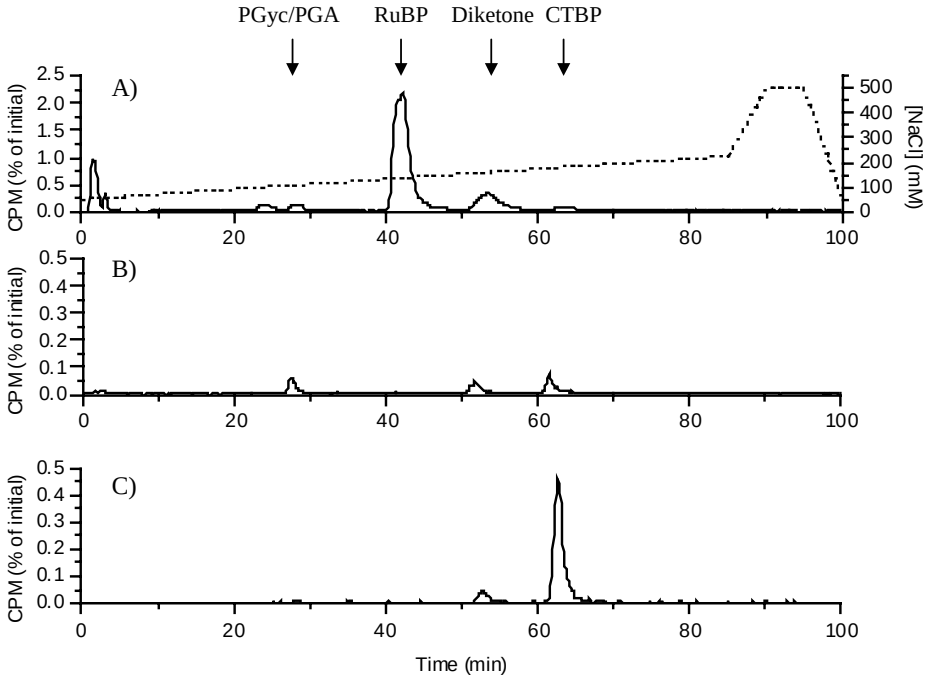


Figure 2. Binding of RuBP derivatives to Rubisco. A) Sample of [^{3}H]-RuBP oxidised with Cu^{2+}, including NaCl gradient (----), B) separation of compounds released from Leu-335 Rubisco with SDS, C) separation of compounds released from Val-335 Rubisco with SDS.

Conclusion

Loop 6 is involved in coordinating the substrate in the active site. Substitution of the Leu residue with Val alters the configuration of the active site, and so alters the kinetic parameters. The binding of RuBP is altered, as are the catalytic abilities and the specificity for CO_2 and O_2. There is also a reduction in the fallover behaviour of the enzyme. This reduction in fallover is not due to a lack of inhibitor binding, as CABP and pentodiulose bisphosphate still bind tightly to the enzyme, with a concurrent loss of activity. The alteration of loop 6 also allows the rearrangement of the diketone to CTBP to occur, without dissociation.

The reduction in fallover in the Val-335 form of Rubisco may be due to an alteration of the binding and catalytic abilities of the enzyme that reduces the pool of free enzyme capable of binding inhibitors during catalysis.

References

Edmondson, D. L., M. R. Badger, *et al*. (1990). *Plant Physiol.* **93**: 1376-1382.
Harpel, M. R., E. H. Serpersu, *et al*. (1995). *Biochemistry* **34**: 11296-11306.
Kane, H. J., J. M. Wilkin, *et al*. (1998). *Plant Physiol.* **117**: 1059-1069.
Lan, Y. and K. A. Mott (1991). *Plant Physiol*. **95**: 604-609.
Lilley, R. M. & Walker, D. A. (1974). *Biochem. Biophys. Acta.* **358**: 226-229
Servaites, J.C. (1985) *Arch. Biochem. Biophys*. **238**: 154-160
Whitney, S. M., S. von Caemmerer, *et al*. (1999). *Plant Physiol.* **121**(2): 579-588.