Volume 233, number 2, 413–416 FEB 05981 June 1988

Release of the nocturnal inhibitor, carboxyarabinitol-1-phosphate, from ribulose bisphosphate carboxylase/oxygenase by rubisco activase

Simon P. Robinson*+ and Archie R. Portis, jr*

*United States Department of Agriculture, Agricultural Research Service, Department of Agronomy, University of Illinois, Urbana, IL 61801, USA and +CSIRO, Division of Horticulture, Adelaide, South Australia, Australia

Received 14 April 1988

Carboxyarabinitol-1-phosphate (CA1P) is a naturally occurring inhibitor of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) which appears in the leaves of some plants during darkness and occupies the ribulose-1,5-bisphosphate binding site. The involvement of the rubisco activase protein in the regulation of rubisco activity by CA1P was investigated. Addition of ribulose bisphosphate (RuBP) or 6-phosphogluconate partially reversed CA1P inhibition by displacing the inhibitor from rubisco. Rubisco activase also partially reversed the inhibition but rubisco activase and RuBP together almost completely reversed CA1P inhibition of rubisco. Rubisco activase did not metabolize CA1P. Inhibition of rubisco by carboxyarabinitol-1,5-bisphosphate was not reversed by rubisco activase in the presence or absence of RuBP. The data suggest that rubisco activase interacts with rubisco to alter the RuBP binding site, thereby increasing the dissociation rate of bound CA1P from the enzyme.

Carboxyarabinitol-1-phosphate; Ribulose bisphosphate carboxylase; Rubisco activase; Photosynthesis

1. INTRODUCTION

Rubisco catalyzes the first step of both photosynthetic carbon reduction and photorespiratory carbon oxidation. Light activation of rubisco occurs in leaves and is thought to involve carbamylation of an inactive form of the enzyme in the presence of CO_2 and Mg^{2+} at alkaline pH [1]. Recently, a naturally occurring inhibitor of rubisco has been found in the leaves of some plant species [2,3]. The inhibitor, which has been identified as CA1P, is structurally similar to the carboxylase reaction intermediate of rubisco and binds to the RuBP site on the activated enzyme forming a stable rubisco-inhibitor complex [4,5]. The level of CA1P is high in the dark and declines in the light suggesting that this represents an additional regulatory mechanism for rubisco in those plants that contain the inhibitor [2,3]. It is not yet known how CA1P is synthesized and metabolized and what factors regulate these processes and hence the concentration of inhibitor in leaves. In vitro, rubisco activase mediates activation of rubisco at physiological levels of CO_2 and RuBP [6]. From studies of a rubisco activase-deficient mutant of *Arabidopsis*, the protein is known to be required for light activation of rubisco in leaves [7]. Although *Arabidopsis* does not contain significant levels of CA1P, rubisco activase has been detected in all higher plants investigated, including some which do accumulate high levels of the inhibitor in the dark [8]. We have investigated the possible involvement of the rubisco activase protein in CA1P inhibition of rubisco, and find that it mediates release of the inhibitor from rubisco but is not involved in its metabolism.

Correspondence address: A.R. Portis, jr, USDA/ARS, S-215 Turner Hall, University of Illinois, 1102 South Goodwin Ave., Urbana, IL 61801, USA

Abbreviations: CA1P, 2-carboxy-D-arabinitol-1-phosphate; CABP, 2-carboxy-D-arabinitol-1,5-bisphosphate; RuBP, ribulose 1,5-bisphosphate; rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; PEP, phospho*enol*pyruvate

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

2. MATERIALS AND METHODS

RuBP was synthesized enzymically from ribose-5-phosphate and purified as described [9]. CA1P, synthesized from RuBP [5], was a kind gift from Dr Joe Berry, Carnegie Institution, Stanford, CA. Rubisco was purified as described [10], then inactivated by filtration through Sephadex (mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the US Dept of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable) G50 equilibrated with 20 mM Tricine, 0.2 mM Tricine, 0.2 mM EDTA, pH 8.0. The enzyme was activated by incubation at 25°C for 1 h in reaction buffer (100 mM Tricine, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 2 mM dithiothreitol, pH 8.0) at a protein concentration of 1–2 $mg \cdot ml^{-1}$. The activated rubisco was then incubated for further 30 min at 25°C with CA1P at 2–4 times the rubisco active site concentration, then diluted 5-fold with reaction buffer (fig.1, table 1) or gel filtered (fig.2) to remove excess CA1P. The inhibited rubisco was incubated at 25°C in reaction buffer and at intervals 50 μl samples were withdrawn and assayed for activity by addition of 450 μl reaction buffer containing 0.5 mM RuBP and 0.3 $Ci \cdot mol^{-1}$ of $^{14}CO_2$. The reaction was terminated after 30 s by adding 10 μl of 4 N formic acid/1 N HCl, the samples dried, and incorporation of ^{14}C into acid stable products determined by liquid scintillation spectroscopy. Rubisco activase was purified from spinach leaves as described [11]. Rubisco protein was determined spectrophotometrically using $A_{280} \times 0.61 = mg \cdot ml^{-1}$ [12] and rubisco activase protein by the dye binding assay.

3. RESULTS

The activated rubisco had a specific activity of 1.4–1.5 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein. Incubation of the enzyme with CA1P in excess of the rubisco active site concentration resulted in more than 95% inhibition of activity. The enzyme-inhibitor complex was stable and the inhibition was not reversed when the rubisco was diluted and incubated at 25°C (fig.1). Addition of RuBP or the positive effector 6-phosphogluconate caused a gradual increase in rubisco activity and after 10–12 min the specific activity reached 0.4–0.6 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ (fig.1). When the enzyme-inhibitor complex was gel-filtered to remove unbound CA1P, there was a slight increase in activity, but again the complex was stable when incubated at 25°C and addition of RuBP led to a slow increase in activity (fig.2). In the absence of RuBP, addition of rubisco activase increased rubisco activity, but there was a much greater increase when both RuBP and rubisco activase were added together (fig.2). Both the initial rate of increase of rubisco activity and the final extent to which the inhibition was

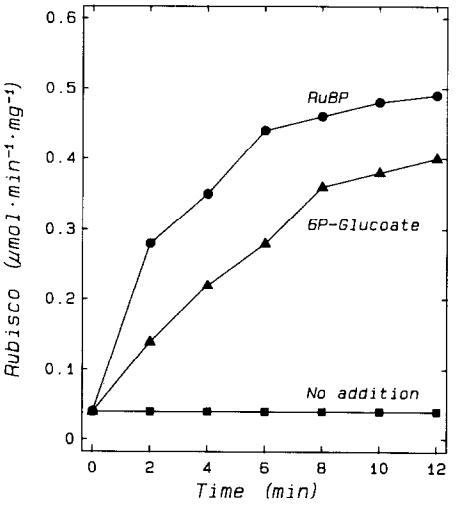

Fig.1. Reversal of CA1P inhibition of rubisco by RuBP and 6-phosphogluconate. Activated rubisco (0.95 $mg \cdot ml^{-1}$, specific activity 1.45 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) was incubated for 30 min at 25°C with 50 μM CA1P, then at zero time diluted 5-fold into reaction buffer (no addition) or buffer with 4 mM RuBP or 1 mM 6-phosphogluconate as indicated. Rubisco activity was measured at the times indicated.

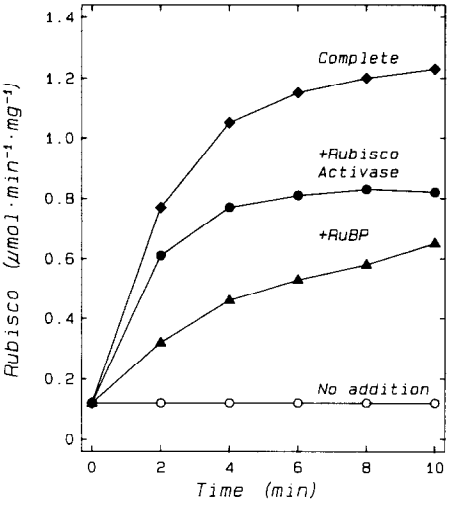

Fig.2. Reversal of CA1P inhibition of rubisco by rubisco activase. Activated rubisco (1.7 $mg \cdot min^{-1} \cdot ml^{-1}$) was incubated for 30 min at 25°C with 77 μM CA1P then gel-filtered through Sephadex G50 equilibrated with reaction buffer to remove unbound CA1P. At zero time, the eluted rubisco was added to reaction buffer (no addition) or to buffer with 3 mM PEP, 1 mM ATP and 20 $U \cdot ml^{-1}$ pyruvate kinase plus 4 mM RuBP (+RuBP), 100 $\mu g \cdot ml^{-1}$ rubisco activase (+rubisco activase) or both RuBP and rubisco activase (complete). Rubisco activity was measured at the times indicated.

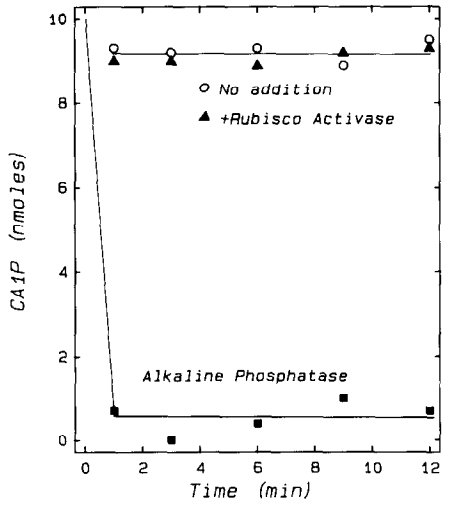

Fig.3. Metabolism of CA1P by alkaline phosphatase but not by rubisco activase. CA1P (10 nmol) was incubated at 25°C for the times indicated in reaction buffer with 5 $U \cdot ml^{-1}$ alkaline phosphatase or with 3 mM PEP, 1 mM ATP, 20 $U \cdot ml^{-1}$ pyruvate kinase, with and without 100 $\mu g \cdot ml^{-1}$ rubisco activase. The samples were boiled for 5 min and the CA1P remaining was determined by titration with rubisco.

reversed were dependent on the concentration of rubisco activase added (data not shown). With 100 $\mu g \cdot ml^{-1}$ rubisco activase protein, rubisco was restored to 85% of the original activity following 10 min incubation with RuBP (fig.2). The reversal of CA1P inhibition by rubisco activase required ATP and an ATP regenerating system, as does the rubisco activase-mediated activation of rubisco in the presence of RuBP [11]. To determine whether CA1P was metabolized by rubisco activase, the inhibitor was incubated with either alkaline phosphatase, which is known to hydrolyze CA1P and reverse its inhibition of rubisco [5], or with ATP in the presence and absence of rubisco activase. After varying times the samples were boiled to stop enzyme activity and then the amount of CA1P remaining was determined by titration with rubisco [5]. With alkaline phosphatase virtually all the inhibitor was hydrolyzed, whereas more than 90% of the added CA1P was recovered after incubation with ATP, both in the presence and absence of rubisco activase. Therefore, no evidence for metabolism was observed (fig.3). CABP is also a potent inhibitor of rubisco and binds more tightly to the enzyme than CA1P. Thus

Table 1

Effect of rubisco activase on rubisco inhibited by CA1P, CABP or RuBP

Form of rubisco	Rubisco activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)		
	No addition	Complete	Minus rubisco activase
Rubisco-CA1P	0.04	0.90	0.46
Rubisco-CABP	0.007	0.020	0.007
Rubisco-RuBP	0.27	1.09	0.05

Inactive rubisco (1 $mg \cdot ml^{-1}$) was incubated 30 min at 25°C with 0.5 mM RuBP or activated and then incubated with 50 μM CA1P or 15 μM CABP. Samples were diluted 5-fold into reaction buffer (no addition) or the same buffer with 3 mM PEP, 1 mM ATP, 20 $U \cdot ml^{-1}$ pyruvate kinase, 4 mM RuBP without (minus rubisco activase) or with (complete) 100 $\mu g \cdot ml^{-1}$ rubisco activase. Rubisco activity was determined after incubation for 6 min at 25°C

CABP inhibition is not reversed by alkaline phosphatase, even though free CABP is readily hydrolyzed, and CABP can displace CA1P from rubisco, whereas CABP cannot be displaced by CA1P [2,5]. The effect of RuBP and rubisco activase plus RuBP on the rubisco-CA1P, rubisco-CABP and inactive rubisco-RuBP complexes is shown in table 1. All three complexes had low rubisco activity in the absence of rubisco activase. In the presence of RuBP (minus rubisco activase) activity was decreased with rubisco-RuBP but increased with rubisco-CA1P. Addition of rubisco activase and RuBP together increased rubisco activity to a similar extent with both the rubisco-CA1P and rubisco-RuBP complexes, whereas neither RuBP alone nor RuBP plus rubisco activase had any marked effect on the activity with rubisco-CABP.

4. DISCUSSION

Binding of CA1P to rubisco is reversible, as evidence by its release with successive precipitations of rubisco and the reversal of CA1P inhibition by alkaline phosphatase or addition of CABP [2]. The slow reversal of rubisco inhibition observed with RuBP or 6-phosphogluconate (fig.1) would be consistent with their competition with free CA1P for binding to the active site on rubisco, effectively displacing CA1P. Since rubisco activase

reversed the inhibition by CA1P (fig.2) but did not metabolize the inhibitor (fig.3), it probably promotes release of CA1P from the active site of rubisco. This would explain the enhanced reversal of inhibition in the presence of both RuBP and rubisco activase (fig.2). The binding constant (K_d) for CABP (less than 10 pM [14]) is much lower than the K_d for CA1P (32 nM [5]) or for RuBP binding to the inactive form of rubisco (21 nM [15]). Thus it is not surprising that neither RuBP nor rubisco activase reversed CABP inhibition of rubisco (table 1).

Berry et al. [5] measured the kinetics of reversal of CA1P inhibition in the presence of saturating levels of alkaline phosphatase and determined a half time of 6.2 min for the spontaneous dissociation of CA1P from rubisco. In the presence of rubisco activase, the half time for reversal of CA1P inhibition was 1.5–2.0 min (fig.2) suggesting that rubisco activase significantly increased the dissociation rate of the rubisco-CA1P complex. The observation that rubisco activase promoted release of CA1P from the activated form of rubisco as well as mediated activation of the inactive rubisco-RuBP complex (table 1) suggests that it may interact with rubisco to alter the RuBP binding site.

Since rubisco activase does not metabolize CA1P, there must be additional proteins in the chloroplast which convert CA1P into a non-inhibitory metabolite in the light. Recent studies have shown that tobacco chloroplasts contain a soluble protein which metabolizes CA1P in the presence of NADPH [16]. Thus light-dependent reversal of CA1P inhibition in vivo may require rubisco activase dependent on ATP, to promote release of the inhibitor from rubisco and another soluble protein dependent on NADPH to metabolize it. In the dark, rubisco activase would be inactivated by the low ATP/ADP ratio [11], allowing CA1P to bind to rubisco and the low NADPH concentration could prevent metabolism of any free CA1P. This would provide a mechanism to coordinate rubisco activity with the light reactions of photosynthesis in those plant species which contain this endogenous nocturnal inhibitor of rubisco.

Acknowledgement: Supported in part by USDA OGPS grant no.86-CRCR-1-2017 to A.R.P.

REFERENCES

[1] Miziorko, H.M. and Lorimer, G.H. (1983) Annu. Rev. Biochem. 52, 507–535.
[2] Seemann, J.R., Berry, J.A., Freas, S.M. and Krump, M.A. (1985) Proc. Natl. Acad. Sci. USA 82, 8024–8028.
[3] Servaites, J.C., Parry, M.A.J., Gutteridge, S. and Keys, A.J. (1986) Plant Physiol. 83, 1161–1163.
[4] Gutteridge, S., Parry, J.A.A., Burton, S., Keys, A.J., Mudd, A., Feeney, J., Servaites, J.C. and Pierce, J. (1986) Nature 324, 274–276.
[5] Berry, J.A., Lorimer, G.H., Pierce, J., Seemann, J.R., Meek, J. and Freas, S. (1987) Proc. Natl. Acad. Sci. USA 84, 734–738.
[6] Portis, A.R., Salvucci, M.E. and Ogren, W.L. (1986) Plant Physiol. 82, 967–971.
[7] Salvucci, M.E., Portis, A.R. and Ogren, W.L. (1986) Plant Physiol. 80, 655–659.
[8] Salvucci, M.E., Werneke, J.M., Ogren, W.L. and Portis, A.R. (1987) Plant Physiol. 84, 930–936.
[9] Jordan, D.B. and Ogren, W.L. (1981) Plant Physiol. 67, 237–245.
[10] Salvucci, M.E., Portis, A.R. and Ogren, W.L. (1986) Anal. Biochem. 153, 97–101.
[11] Robinson, S.P., Streusand, V.J., Chatfield, J.M. and Portis, A.R. (1988) Plant Physiol., submitted.
[12] Wishnick, M. and Lane, M.D. (1971) Methods Enzymol. 23, 570–577.
[13] Spector, T. (1978) Anal. Biochem. 86, 142–146.
[14] Pierce, J., Tolbert, N.E. and Barker, R. (1980) Biochemistry 19, 934–942.
[15] Jordan, D.B. and Chollet, R. (1983) J. Biol. Chem. 258, 13752–13758.
[16] Salvucci, M.E., Holbrook, G.P., Anderson, J.C. and Bowes, G. (1988) FEBS Lett. 231, 197–201.