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REGULATION OF ACTIVATION OF RIBULOSE BISPHOSPHATE CARBOXYLASE FROM PSEUDOMONAS OXALATICUS

V. Bryan Lawlis, Geoffrey L. R. Gordon and Bruce A. McFadden
Program in Biochemistry and Biophysics, Department of Chemistry,
Washington State University, Pullman, WA 99164

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SUMMARY: 6-phosphogluconate, potentiated activation of ribulose bisphosphate carboxylase from Pseudomonas oxalaticus whereas fructose-1,6-bisphosphate inhibited activation and fructose-6-phosphate had no effect. The presence of 1 mM 6-phosphogluconate during activation reduced the K_{act} for Mg^{2+} from 1.4 mM to approximately 0.2 mM. In the absence of 6-phosphogluconate, the enzyme responded sigmoidally to increasing CO_2 (Hill coefficient, h, of 1.8), with a concentration causing half maximal activation, $Act_{0.5}$, of 15 mM $NaHCO_3$. In the presence of 1 mM 6-phosphogluconate h was reduced to 1.1 and an $Act_{0.5}$ value of 5 mM $NaHCO_3$ was obtained. 6-phosphogluconate appeared to saturate at or below 20 μmM.

INTRODUCTION: For many years it was evident that both plant and bacterial D-ribulose-1,5-bisphosphate* carboxylases (E.C. 4.1.1.39) were optimally active only when incubated with Mg^{2+} and HCO_3^- prior to initiation of catalysis by RuBP (1-4). In 1976 this requirement was clarified by Lorimer et al., who established that the spinach enzyme acquired catalytic activity only after sequential binding of CO_2 and Mg^{2+} (5). Thus it became clear that catalysis was the result of two phenomena: 1). activation and 2). catalytic turnover of substrates.

The regulation of RuBP carboxylase may be of considerable importance in influencing CO_2 assimilation via the reductive pentose phosphate cycle in autotrophic species. Although it will be ultimately important to simulate this regulation under "physiological conditions," it is crucial as a first step to design experiments that distinguish between the action of effectors upon activation and catalysis. We now describe effects of the metabolite 6-phosphogluconate upon activation of RuBP carboxylase from Pseudomonas oxalaticus by Mg^{2+} and HCO_3^- under conditions when these latter ligands saturate with respect to catalysis.

MATERIALS AND METHODS: Pseudomonas oxalaticus was grown aerobially at 30° C on 100 mM formate (6). RuBP carboxylase was purified from thawed cells as generally described elsewhere (7) to yield a preparation with a final specific activity of

*Abbreviation: RuBP = D-ribulose-1,5-bisphosphate

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1.5 units/mg. The purified enzyme was stored at 2° to 5° C in 50 mM HEPES, pH 8.0, containing 20 mM $MgCl_2$, 50 mM $NaHCO_3$, 1 mM EDTA and 5 mM 2-mercaptoethanol, and retained high activity for two weeks. Protein was determined colorimetrically (8) using crystalline bovine serum albumin as a standard.

"CO_2 free"-N_2 was prepared by sparging N_2 gas through a saturated solution of $Ca(OH)_2$. This N_2 was used in all subsequent experiments which required a N_2 atmosphere. "CO_2-free" 100 mM Tris-Cl (pH 8.0, 23° C) was prepared with glass-distilled water which had been acidified to pH 2 to 3, boiled, cooled and stored under N_2.

Inactivation of RuBP carboxylase was performed by filtration into the Tris buffer using Sephadex G-25 gel at 23° C essentially as described by Lorimer et al. (5). Tests for reactivation were conducted at 30° C for 5 minutes under a N_2 atmosphere by incubating enzyme solution with an equal volume of solution containing various indicated combinations of $NaHCO_3$, $MgCl_2$ and organic phosphates. The water used to prepare these solutions had been prepared and stored as described for "CO_2-free" buffer. However, no attempt was made to remove Mg^{2+} from the water.

RuBP carboxylase activity was measured (9) by adding 50 µl of the enzyme preparation after the 5-minute incubation to 200 µl of an assay mixture which contained in a final volume of 250 µl: 64 mM Tris-Cl (pH 8.0, 23° C), 8 mM $MgCl_2$, 20 mM $NaH^{14}CO_3$ (0.3 Ci/mole) and 0.8 mM RuBP. Reactions were conducted at 30° C for 15 seconds and terminated with 100 µl of 60% cold trichloroacetic acid. Excess $^{14}CO_2$ was liberated from a 200 µl sample by heating at 70-85° C for 45 minutes prior to liquid scintillation counting. Controls to test the inhibitory effect of 6-phosphogluconate (or other organic phosphates) carried over into the assay were routinely conducted.

RESULTS: When RuBP carboxylase from P. oxalaticus was inactivated and subsequently incubated either in the absence of added Mg^{2+} and/or HCO_3^- and then assayed, an initial rate of 2-5% of that of the fully activated control was observed (Table 1). This result indicated that gel filtration under N_2 had generated enzyme relatively free of Mg^{2+} and CO_2.

Table 1 also demonstrates that 6-phosphogluconate at 1.0 mM will not substitute for Mg^{2+} or CO_2 in the activation of RuBP carboxylase. However, with the addition of low levels of both Mg^{2+} and HCO_3^- activation was greatly enhanced. Indeed whereas 5 mM HCO_3^- plus 1.0 mM Mg^{2+} or 10 mM Mg^{2+} activated 15% and 53%, respectively, these values were increased to 68 and 89%, respectively, in the presence of 1.0 mM 6-phosphogluconate. The intermediate fructose-1,6-bisphosphate inhibited activation at 1.0 mM in the range of 36-52% depending upon the concentrations of Mg^{2+} and HCO_3^- whereas another intermediate, fructose-6-phosphate, had no effect at 1 mM. Inhibition of catalysis by these compounds at 0.2 mM was slight at saturating substrates and Mg^{2+}.

Table 1. Effect of 6-Phosphogluconate (PGN) on Activation of RuBP Carboxylase from P. oxalaticus

Activating Conditions		RuBP Carboxylase*			
$MgCl_2$ (mM)	$NaHCO_3$ (mM)	No PGN	$PGN^{**}_{a,c}$	PGN_c	$PGN_{a,c}/PGN_c$
0	0	___	1.6	0.6	2.7
1	0	___	6.0	0.9	6.7
0	5	___	6.8	1.3	5.2
1	5	___	23.3	3.6	6.5
1	50	___	35.3	25.3	1.4
10	5	___	30.6	13.0	2.4
10	50	38.2	34.3	24.6	1.4

*Nmoles CO_2 fixed min^{-1}. The specific activity of maximally activated enzyme was 1.5 u mg^{-1} and 25 µg protein were used per assay.

**6-Phosphogluconate (1 mM) was present in the activation mixtures and subsequently 0.2 mM was carried over to the assays ($PGN_{a,c}$); therefore controls were conducted which contained only 0.2 mM 6-phosphogluconate in the assay (PGN_c).

Figure 1 demonstrates the effect of 6-phosphogluconate on activation when the concentration of Mg^{2+} was varied and HCO_3^- was maintained at saturating levels. These data suggest that 1 mM 6-phosphogluconate has very little effect on the maximal state of activation of the enzyme. However, 6-phosphogluconate appeared to decrease the K_{act} for Mg^{2+} (concentration of Mg^{2+} resulting in half maximal activation) from 1.4 mM to approximately 0.2 mM (7-fold). The alternative interpretation that Mg^{2+} present in the 6-phosphogluconate preparation may have accounted for the decrease observed in $K_{act}^{Mg^{2+}}$ was ruled out because Mg^{2+} present in the same preparation was found to contribute 1.0 µM (by atomic absorption) to the preincubation.

When HCO_3^- concentration in the activating mixture was varied while holding Mg^{2+} at saturating levels (Figure 2A), a sigmoidal curve was observed with a Hill

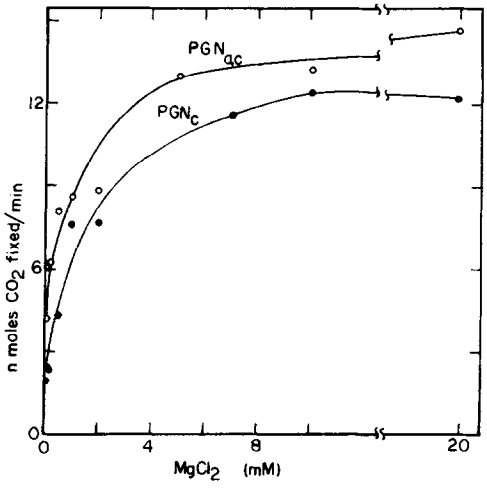

Figure 1. Effect of 6-phosphogluconate on the activation of P. oxalaticus RuBP carboxylase by $MgCl_2$. A "CO_2-free" enzyme preparation (specific activity: 1.4 u mg^{-1}) was incubated at 30° C for 5 minutes in the presence of 50 mM $NaHCO_3$ plus 1 mM 6-phosphogluconate prior to 15-second assay ($PGN_{a,c}$). As a control, samples of the enzyme incubated in 50 mM $NaHCO_3$ as above were assayed for 15 seconds in reaction mixtures containing 0.2 mM 6-phosphogluconate (PGN_c), a concentration equivalent to that carried over from activation mixtures. The protein concentration of the activation mixtures was 0.26 mg ml^{-1} while 13 µg protein was used in each assay.

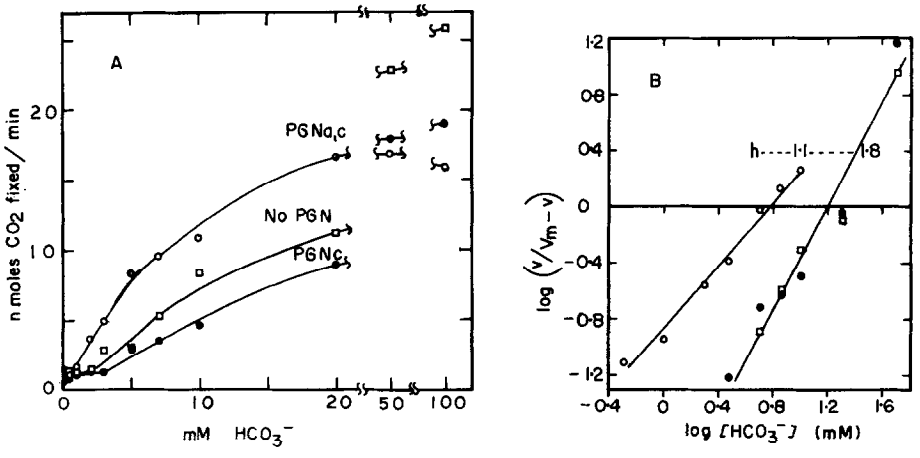

Figure 2A. Effect of 6-phosphogluconate on the activation of P. oxalaticus RuBP carboxylase by $NaHCO_3$. The "CO_2-free" enzyme preparation (Spec. Act. 1.0 u mg^{-1}) was incubated with 20 mM $MgCl_2$ and 1 mM 6-phosphogluconate ($PGN_{a,c}$). A control with 0.2 mM 6-phosphogluconate in the assay was included (PGN_c). The protein concentration of the activation mixtures was 0.5 mg ml^{-1} while 25 µg protein was used in each assay. Figure 2B. Hill plots for the bicarbonate activation curves given in Figure 2A. Symbol: h, Hill coefficient (obtained by linear least squares analysis).

coefficient of 1.8 (Figure 2B). However, upon the addition of 1.0 mM 6-phosphogluconate the Hill coefficient became 1.1 (Figure 2B) and the concentration of HCO_3^- which half maximally activated decreased from 15 mM to 5 mM. Again 6-phosphogluconate altered the maximal state of activation slightly, if at all.

Attempts were made to measure saturating concentrations of 6-phosphogluconate with respect to activation in the presence of suboptimal concentrations of Mg^{2+} and HCO_3^-, 1 and 5 mM, respectively. In the range of 0.02-1 mM 6-phosphogluconate the initial rate of catalysis was constant (6-fold greater than that for the control in the absence of phosphogluconate). This rate in turn was about 30% of that achieved with fully activated enzyme. These data suggest that under these conditions, 6-phosphogluconate saturates the enzyme at or below 20 μM.

DISCUSSION: In a recent review, Jensen and Bahr have summarized the kinetic parameters for RuBP carboxylase (10). In particular the K_m for CO_2 for the catalytic reaction which we shall refer to as $K_{cat}^{CO_2}$ was 6-21 μM for the higher plant enzymes. It has been difficult to estimate the analogous $K_{cat}^{Mg^{2+}}$ but it is much less than 15 and 40 μM for the soybean enzyme (11) and the enzyme from the photosynthetic bacterium Thiocapsa roseopersicina, respectively (K. Purohit and B. A. McFadden, unpublished observation). Indeed it is possible that Mg^{2+} is not required for catalysis but only for activation. Considering these data obtained with plant and bacterial RuBP carboxylases and the fact that this enzyme has been strongly conserved during evolution (12,13), it is likely that in the present studies both Mg^{2+} and HCO_3^- were saturating at 10 and 50 mM (about 1.7 mM CO_2) in addition to RuBP (furnished at 3 x the K_m) with respect to catalysis. Only initial incorporation of $H^{14}CO_3^-$ was measured during an extremely short 15-second interval of catalysis in these studies. The carryover of inhibitory 6-phosphogluconate was duplicated in controls. Thus in the present work it is virtually certain that the action of effectors upon the P. oxalaticus enzyme was on activation and not catalysis.

In the present paper, the potentiation of activation by Mg^{2+} and CO_2 exerted by 6-phosphogluconate has been described. In particular 1 mM 6-phosphogluconate reduces

the K_{act} values for Mg^{2+} and CO_2 by 3-7 fold and eliminates ostensibly cooperative binding of CO_2. The effect on $K_{act}^{Mg^{2+}}$ is evident in the concentration range of 0.1-0.5 mM Mg^{2+}. Of further significance is the fact that the effect of 6-phosphogluconate saturates below 20 μM, at least at one pair of Mg^{2+} and CO_2 concentrations. Because 6-phosphogluconate is a linear competitive inhibitor with respect to RuBP (K_i = 0.20 mM; unpublished observation) of RuBP carboxylase from P. oxalaticus at saturating HCO_3^- and Mg^{2+}, the two opposing effects of 6-phosphogluconate suggest two classes of binding sites--the active site and one for activation--confirming an earlier suggestion (14).

In research that may be related, Chu and Bassham reported that 6-phosphogluconate enhanced the activity of spinach RuBP carboxylase when the ligand was present at low concentrations during preincubation of the enzyme with 10 mM Mg^{2+} and 1 mM HCO_3^- (14-16). Some enhancement was also observed with fructose bisphosphate (14). Non-linear double-reciprocal plots reflecting rate dependence upon [HCO_3^-] became linear as the result of the presence of 6-phosphogluconate (14). These investigators could only observe inhibition by 6-phosphogluconate (17,18) when it was added simultaneously with or later than RuBP or it was tested at 50 mM HCO_3^- (14,15). On the basis of the present research it is likely that the enhancement of the activity by phosphogluconate observed by Chu and Bassham (14-16) reflected the influence of this ligand upon activation of the plant enzyme.

In conclusion, the present data suggest that 6-phosphogluconate, which is present in chloroplasts in the light, and which can be formed from glucose-6-phosphate in P. oxalaticus (G. L. R. Gordon and B. A. McFadden, unpublished observation), has a significant role at low concentrations in decreasing the [CO_2] and [Mg^{2+}] required for activation of RuBP carboxylase. The latter effect is prominent in the concentration range of 0.1-0.5 mM, at least with the bacterial enzyme. Whether 6-phosphogluconate, which accumulates in green algae in the dark (19) and perhaps during heterotrophic metabolism (17), contributes to the onset of RuBP carboxylase inhibition in the transition from autotrophic to heterotrophic metabolism remains to be seen.

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