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ACTIVATION AND INHIBITION OF SPINACH RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE BY 2-PHOSPHOGLYCOLATE

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach; ribulose 1,5-bisphosphate carboxylase; phosphoglycolate; inhibition; product activation.

Abstract—Ribulose 1,5-bisphosphate carboxylase when activated by preincubation with 1 mM bicarbonate and 10 mM magnesium chloride can be further activated *ca* 20–500% by incubating with 2.5 mM phosphoglycolate depending upon the pH of the preincubation medium. The activation effects were seen only under specific preincubation conditions. The activation by phosphoglycolate was a slow reaction requiring *ca* 15 min for maximal effect. Even though magnesium was essential for phosphoglycolate activation, concentrations higher than 15 mM progressively inhibited the activation of the enzyme by phosphoglycolate. When added directly to the reaction mixture, phosphoglycolate was a potent inhibitor of the carboxylase activity. Even under preincubating conditions, phosphoglycolate showed slight inhibitory effect at 0.1 mM and activation was observed at concentrations higher than 0.5 mM. The K_A value for phosphoglycolate was 2.8 mM.

INTRODUCTION

Ribulose 1,5-bisphosphate carboxylase (EC 4.1.1.39) in all photosynthetic organisms so far examined catalyses the carboxylation and oxygenation of RuBP [1, 2]. Both reactions seem likely to be subject to metabolic regulation *in vivo*, since they are accompanied by a high negative free energy change under conditions of steady state photosynthesis [3, 4]. Recent studies have pointed out that the activity of this enzyme is profoundly affected by HCO_3^- concentration, and the order of addition of the substrates and effectors. It is also known that RuBP carboxylase contains a number of subunits and many binding sites for substrates [5–8]. The effect of several chloroplast metabolites on RuBP carboxylase/oxygenase activities have been reported [9–16].

This report describes the effect of 2-phosphoglycolate, a product of RuBP oxygenase reaction, on RuBP carboxylase activity. The results presented here show that phosphoglycolate can both activate and inhibit spinach RuBP carboxylase under specific conditions. The response of this enzyme to this effector was apparently quite complex, like many other effectors, as it is greatly influenced by bicarbonate concentration, pH and order of addition of substrates and effectors.

RESULTS

Activation of RuBP carboxylase by phosphoglycolate. Phosphoglycolate was an activator of both carboxylase and oxygenase reactions when the enzyme was preincubated with phosphoglycolate under conditions of low bicarbonate in the presence of Mg^{2+}. It was found that dissolved CO_2 in buffer solutions could activate the enzyme to different degrees depending on the buffer system. It was, therefore, necessary to make buffer solutions CO_2-free so that the activation effects due to preincubation with HCO_3^- and Mg^{2+} could be determined accurately. The data presented in Fig. 1 show that when the enzyme was activated with 1 mM HCO_3^- and 10 mM $MgCl_2$ and incubated in the presence of various concentrations of phosphoglycolate, the carboxylase enzyme was activated by 2–2.5-fold. However, the enzyme activated at 10 mM HCO_3^- and 10 mM $MgCl_2$ showed only slight activation on incubation with phosphoglycolate. Low concentrations of phosphoglycolate (0.05 and 0.1 mM) showed inhibitition (15–20%) of the carboxylase activity under preincubating conditions. This observation is supported by the data on inhibition of the enzyme activity by phosphoglycolate to be presented later. The stimulation by phosphoglycolate was seen at concentrations higher than 0.5 mM. Determination of K_A for phosphoglycolate is shown in Fig. 1 (inset); a value of 2.8 mM was obtained.

Effect of preincubation time. The effect of incubation time on the rate of activation was studied at 1 and 2.5 mM phosphoglycolate. The data for 2.5 mM phosphoglycolate presented in Fig. 2 show that the activation by phosphoglycolate was a slow reaction requiring *ca* 15 min to show maximal activation.

The effect of different buffer systems like Tris–HCl–NaOH, Tricine–NaOH, Bicine–KOH and HEPES–KOH on the activation of the enzyme by phosphoglycolate was also studied but no change in

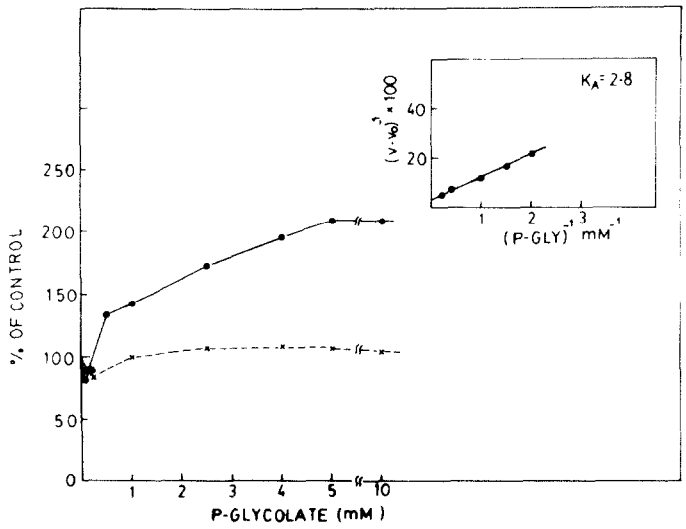

Fig. 1. Effect of phosphoglycolate on RuBP carboxylase activity with preincubation. The enzyme was activated with either 1 or 10 mM HCO_3^- in 100 mM Bicine–KOH buffer pH 7.8 for 20 min. The enzyme samples were subsequently incubated with the indicated concentrations of phosphoglycolate for another 30 min. Enzyme was assayed at the same concentration of HCO_3^- as included in the preincubation. The control without phosphoglycolate was taken as 100%. The reaction mixture in 0.5 ml contained 100 mM Bicine–KOH, pH 7.8, 10 mM $MgCl_2$, 1 or 10 mM $NaH^{14}CO_3$, 0.5 mM RuBP. Specific activity of the enzyme in the absence of effector was 0.07 at 1 mM HCO_3^- and 1.5 at 10 mM HCO_3^-. (●) Preincubated and assayed at 1 mM HCO_3^-; (×) preincubated and assayed at 10 mM HCO_3^-. Inset: determination of K_A for phosphoglycolate activation.

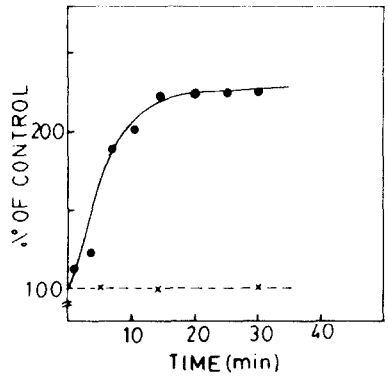

Fig. 2. Effect of incubation time on phosphoglycolate activation. Enzyme activated with 1 mM HCO_3^- and 10 mM $MgCl_2$ in 100 mM Bicine–KOH pH 7.8 was incubated with 2.5 mM phosphoglycolate. Aliquots were withdrawn at the specified intervals and assayed for carboxylase activity. Control without phosphoglycolate was taken as 100%. Control (×); with phosphoglycolate (●).

the extent of activation of the enzyme by phosphoglycolate was observed.

Effect of pH. pH markedly influences the response of the enzyme to phosphoglycolate (Fig. 3). Phosphoglycolate activation was maximal at neutral pH and the activation decreased to 20% at pH 8.9. Since upon illumination of intact spinach chloroplast, the pH of the stroma increases by *ca* one pH unit, from pH 7 to 8, due to proton translocation into thylakoids, and CO_2 concentration would be *ca* 1 mM HCO_3^- at pH 8, the effect of phosphoglycolate should be evaluated at this pH range and bicarbonate concentration.

Effect of bicarbonate. As mentioned earlier, the bicarbonate concentration of the preincubation mixture strongly influenced the activation of the enzyme by phosphoglycolate. The data presented in Fig. 4 show that increasing bicarbonate concentration in the preincubation mixture decreased the activation progressively, and beyond 10 mM HCO_3^- only slight activation was observed. However, even at 25 mM HCO_3^- no inhibition of the enzyme activity was evident during preincubation with either 1 or 2.5 mM phosphoglycolate.

Effect of Mg^{2+}. Sugar phosphates can chelate Mg^{2+} and therefore may affect the activation of the enzyme. Thus it became imperative that the effect of Mg^{2+} concentration on phosphoglycolate activation of the enzyme be studied. The results presented in Fig. 5 show a strict requirement of Mg^{2+} for phosphoglycolate activation. However, increasing the Mg^{2+} concentration beyond 10 mM did not enhance the extent of activation. On the other hand, Mg^{2+} levels in the preincubation mixture above 15 mM resulted in progressive inhibition of the activation of the enzyme.

The enzyme activated with phosphoglycolate showed a K_m (CO_2) similar to that of a control but the V_{max}, however, had increased by *ca* 2-fold (data not included).

Influence of effectors on phosphoglycolate activation. When the enzyme activated with 6-phosphogluconate (6-PGLuA), fructose diphosphate (FDP) or NADPH at 1 mM HCO_3^- and 10 mM $MgCl_2$ was subsequently incubated with various concentrations of phosphoglycolate, it was observed that phospho-

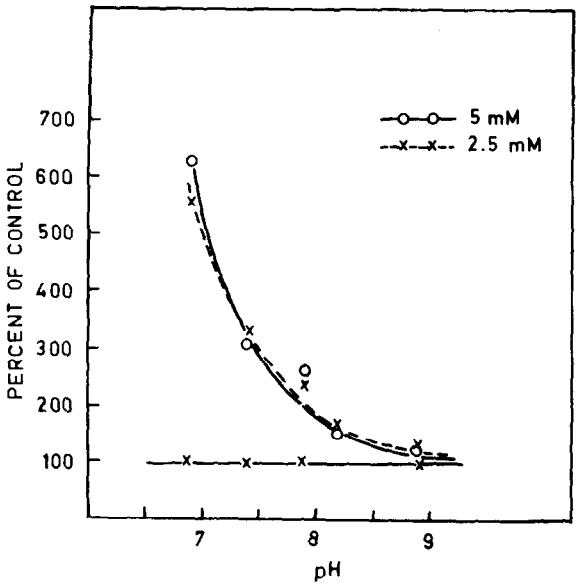

Fig. 3. Effect of *preincubation* pH on phosphoglycolate activation of enzyme. Preincubations were done in 100 mM HEPES–KOH buffer of the indicated pH values (CO_2-free). The preincubation mixture also contained 1 mM HCO_3^-, 10 mM $MgCl_2$ and 2.5 or 5 mM phosphoglycolate. The enzyme was assayed at the same pH that was used for preincubation. Controls contained all the ingredients except phosphoglycolate. The assays were done at low bicarbonate concentrations (1 mM).

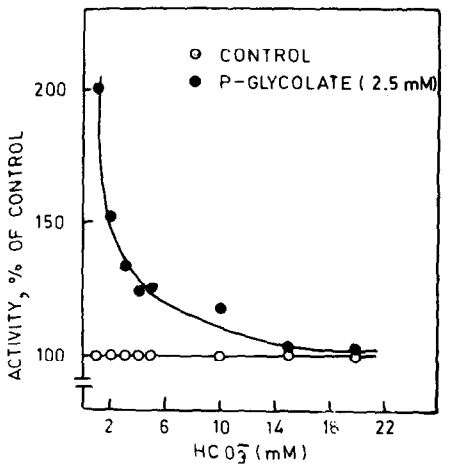

Fig. 4. Effect of bicarbonate concentration on phosphoglycolate activation. The enzyme was activated with the indicated concentration of HCO_3^- and 10 mM $MgCl_2$ for 20 min. The samples were subsequently incubated with 2.5 mM phosphoglycolate for another 30 min. The samples were assayed at the same concentration of HCO_3^-, that was included in the preincubation mixture. Controls without phosphoglycolate were treated identically.

glycolate deactivated the enzyme to some extent. FDP which was found to activate the enzyme by *ca* 25–30% prevented the subsequent activation of the enzyme by phosphoglycolate (Fig. 6).

Inhibition of RuBP carboxylase by phosphoglycolate. Phosphoglycolate was found to be a potent inhibitor of the carboxylase activity without preincubation and when added directly to the reaction mixture before addition of the enzyme. The inhibition studies were done both at 1 mM and 10 mM HCO_3^- concentrations (Fig. 7A and B). The K_i for phosphoglycolate was 2.9 mM. The phosphoglycolate-stimulated enzyme, when assayed at the same concentration of phosphoglycolate that was used for activation, showed a mixed inhibitory response by phosphoglycolate.

As mentioned earlier, low concentrations of phosphoglycolate were inhibitory even under preincubating conditions. This observation was further sub-

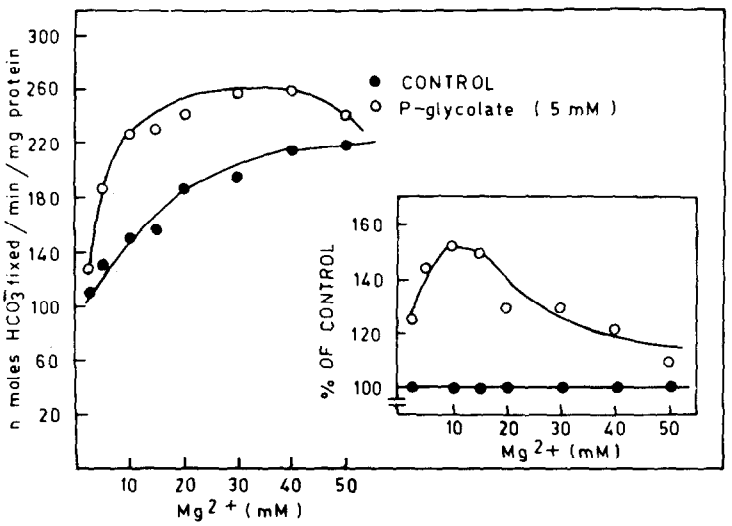

Fig. 5. Effect of magnesium concentration on phosphoglycolate activation of carboxylase activity. The enzyme (3 mg/ml) activated with 1 mM HCO_3^- was incubated with 5 mM phosphoglycolate at the indicated concentrations of Mg^{2+}. After 30 min of preincubation, the enzyme activity was measured at 1 mM HCO_3^-. Controls without phosphoglycolate were treated identically. Inset: a plot of per cent control activity at 2.5 mM phosphoglycolate as a function of Mg^{2+} concentration.

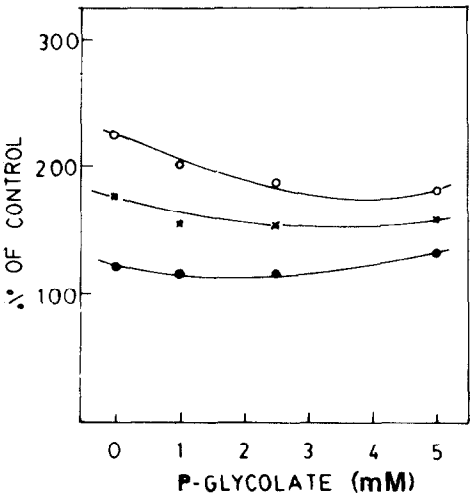

Fig. 6. Influence of different effectors on phosphoglycolate activation of the enzyme. The enzyme (5 μM) was preincubated with 1 mM HCO_3^- and 10 mM $MgCl_2$ at pH 7.8 prior to addition of 0.1 mM phosphoglucinate (×); 0.1 mM fructose diphosphate (●); and 0.1 mM NADPH (○). This was followed by a second incubation with phosphoglycolate concentrations indicated on the abscissa for 30 min. The enzyme activity was measured at 1 mM HCO_3^-.

stantiated by assaying the phosphoglycolate preincubated enzyme at the same concentration of phosphoglycolate as that used for activation. The results presented in Fig. 7A show that an additive inhibitory response of phosphoglycolate was observed at a 0.1 mM concentration. This probably indicates that, at this concentration, the enzyme response to the effector is similar under both conditions of assay. The inhibition of the carboxylase activity by increasing phosphoglycolate concentrations was much less when the phosphoglycolate-activated enzyme was used as compared to the enzyme which was not preincubated with phosphoglycolate.

DISCUSSION

The observed effects of phosphoglycolate on RuBP carboxylase activity are distinctly different from those exerted by other chloroplastic metabolites like 6-PGLuA, FDP, PGA and Ru5P. The major differences are as follows. (a) When used under preincubating conditions, phosphoglycolate was found to be moderately inhibitory at low concentrations (0.1 mM) and activation was observed at concentrations higher than 0.5 mM. The maximal activation was observed at relatively higher concentration of 2.5 mM. (b) No inhibition or decrease in activation was observed even at 10 mM phosphoglycolate during preincubation. (c) Binding of phosphoglycolate is relatively slower as compared to other effectors. The slow response of the enzyme to phosphoglycolate may be an example of the hysteretic phenomenon proposed by Frieden [17]. (d) No inhibition of the carboxylase activity by phosphoglycolate was evident in the concentration range 0.5–10 mM even when the enzyme was preincubated with phosphoglycolate at 10 or 25 mM HCO_3^- and assayed at the same concentration of HCO_3^- without effector.

Laing and Christeller [18] have recently reported the kinetics of inhibition of the enzyme by phosphoglycolate using soyabean RuBP carboxylase. They have shown that the inhibition by phosphoglycolate with respect to RuBP and CO_2 concentrations was competitive and non-competitive, respectively. This observation, along with the results presented here, suggests that phosphoglycolate affects the enzyme activity in two ways: (1) it competes with RuBP at the catalytic site but does not alter the affinity of the enzyme towards bicarbonate. This results in inhibition of the enzyme activity; (2) activation of the carboxylase activity may be due to its effect on the activator site, when preincubated at low HCO_3^- concentration. It is likely that binding of phosphoglycolate to the enzyme results in a conformational change as indicated by the decrease in 6-PGLuA and NADPH stimulation after phosphoglycolate binding.

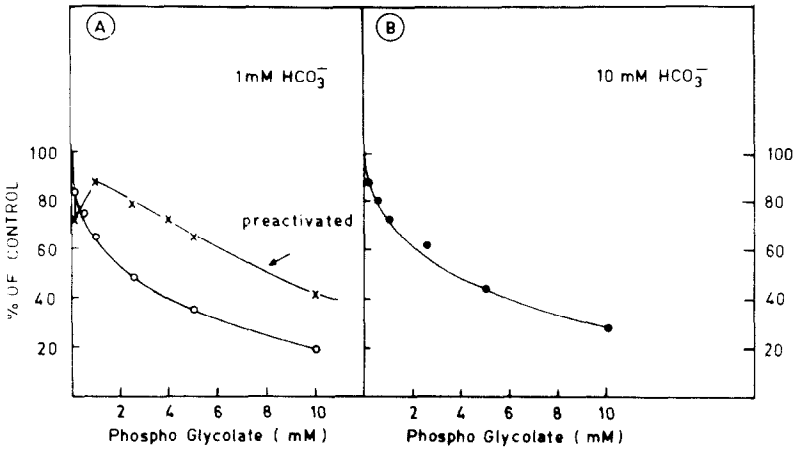

Fig. 7. Inhibition of carboxylase activity by phosphoglycolate in preincubated and non-preincubated conditions. (A) Enzyme activated and assayed at 1 mM HCO_3^-, with (×) and without (○) preincubation at the indicated phosphoglycolate concentrations. (B) Enzyme activated and assayed at 10 mM HCO_3^- without preincubation with phosphoglycolate. The reaction mixture contained the indicated concentrations of phosphoglycolate. Control without phosphoglycolate was considered as 100%.

Regarding the activation of the enzyme by sugar phosphates two possibile mechanisms have been suggested. Lorimer *et al.* [19] have suggested that most phosphate esters may act by altering the pK of the group, most probably lysine, with which CO_2 reacts. Buchanan and Schurman [20] favour the view that activation by several sugar phosphates could be due to lower values for apparent K_m (CO_2), thus resulting in higher activities at low CO_2 concentrations. The data presented in this paper do not support the latter view.

EXPERIMENTAL

RuBP carboxylase was prepared from spinach leaves essentially as described in ref. [21] and was highly purified by the criterion of PAGE. The fully activated enzyme had a sp. act. of 1.5 μmol CO_2 fixed/min/mg protein. All biochemicals were purchased from Sigma Chemical Co. and used without further purification.

All incubations were performed in 0.1 M Bicine–KOH or HEPES–KOH buffer (CO_2-free) pH 7.8 at 25° with noted additions. All buffers and other solns including phosphoglycolate were prepared just before use in freshly distilled or boiled H_2O and the pH was adjusted with freshly prepared KOH soln. All solns except bicarbonate were degassed and flushed with N_2 alternately 2–3 times and kept in sealed tubes. All preincubations were done in sealed tubes. The enzyme soln was passed through a Sephadex G-25 column at room temp. The column was equilibrated with CO_2 free buffer. This step removed residual $(NH_4)_2SO_4$, CO_2 and Mg^{2+} from the enzyme prepn. Incubations with HCO_3^- and Mg^{2+} were performed for *ca* 30 min. When appropriate, the first incubation (HCO_3^- and Mg^{2+}) was followed by a second incubation with effectors other than phosphoglycolate for 10 min. Incubations with phosphoglycolate were performed for 30 min unless otherwise indicated. The carboxylase assays were always initiated by addition of 20 μl of the enzyme and all assays were terminated after 30 sec.

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