Bicarbonate Stabilization of Ribulose 1,5-Diphosphate Carboxylase†

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ABSTRACT: The carboxylase and oxygenase activities of purified soybean ribulose 1,5-di-P carboxylase (EC 4.1.1.39) were unstable when reactions were initiated with enzyme. Time courses of carboxylase and oxygenase activities were curvilinear, approximating hyperbolas. Double reciprocal plots of amount of CO_2 incorporated and P-glycolate produced vs. time were constructed to determine a constant representing the half-time of initial enzyme activity, *K*. *K* increased with increasing bicarbonate concentration but was independent of O_2 tensions between 0.21 and 5 atm. When time courses of carboxylase and oxygenase activities were determined simultaneously, *K* was identical for both activities. Linear time courses were obtained by preincubation of the enzyme for 10 min in the absence of bicarbonate or by adding 46 m*M* $MgCl_2$ to the reaction mixture. The observed bicarbonate-dependent decline in ribulose 1,5-di-P carboxylase activity with time is the probable cause for the anomalously high $K_m(CO_2)$ values previously reported for this enzyme. In the experiments reported here, the apparent $K_m(CO_2)$ at pH 8.5 increased from 6 μM CO_2 at zero time to 78 μM CO_2 at 10 min. The corresponding bicarbonate K_m values are 1.3 and 17 m*M*, respectively. The interaction between bicarbonate and enzyme may be important in the light activation of photosynthetic CO_2 fixation in vivo.

Ribulose 1,5-di-P carboxylase (EC 4.1.1.39) catalyzes the carboxylation (Weissbach et al., 1956) and oxygenation (Bowes et al., 1971) of ribulose 1,5-di-P. CO_2 and O_2 compete with each other on the enzyme for ribulose 1,5-di-P (Ogren and Bowes, 1971; Bowes and Ogren, 1972; Laing et al., 1974). One kinetic property of the enzyme which has evoked considerable interest is the Michaelis constant for CO_2 (see review, Walker, 1973). Although the apparent $K_m(CO_2)$ of the ribulose 1,5-di-P carboxylase is the equivalent of approximately 0.6 m*M* bicarbonate in isolated chloroplasts (Jensen and Bassham, 1966), intact leaf cells (Jensen et al., 1971), and leaves (Goldsworthy, 1968; Akita and Moss, 1973), the apparent $K_m(CO_2)$ for the isolated enzyme ranges from the equivalent of 5 (Ogren and Bowes, 1971; Bowes and Ogren, 1972) to 22 (Paulsen and Lane, 1966) m*M* bicarbonate when the enzyme is assayed in the presence of 5–10 m*M* $MgCl_2$. The apparent $K_m(CO_2)$ is decreased by assaying the enzyme in high (20–45 m*M*) $MgCl_2$ concentrations (Sugiyama et al., 1968a; Bassham et al., 1968) or by assaying the enzyme immediately after breaking intact chloroplasts (Jensen, 1971; Bahr and Jensen, 1974).

In contrast to the order-of-magnitude $K_m(CO_2)$ difference observed for ribulose 1,5-di-P carboxylase in vitro compared to in vivo, the $K_m(O_2)$ in the oxygenation reaction and $K_i(O_2)$ in the carboxylation reaction is in the range of 40–70% O_2 for the purified enzyme (Ogren and Bowes, 1971; Bowes and Ogren, 1972; Laing et al., 1974; Andrews et al., 1973), intact *Chlamydomonous* cells (Bowes and Berry, 1972), and leaves (Forrester et al., 1966; D'Aoust and Canvin, 1973; Ludwig and Canvin, 1971). In this paper, kinetic properties of ribulose 1,5-di-P carboxylase were examined in an attempt to resolve the discrepancy between the in vivo and in vitro $K_m(CO_2)$ of the enzyme.

Materials and Methods

Preparation of Enzymes. Ribulose 1,5-Di-P Carboxylase. This enzyme was isolated from leaves of soybean (*Glycine max* (L.) Merr., var. 'Wayne') as described previously (Bowes and Ogren, 1972), except that 20 g of polyvinylpyrrolidone per 100 g of leaf material was added to the grinding medium, and purification terminated with the DEAE-cellulose step. The purified carboxylase was stored as a precipitate at 3° in 0.15 *M* potassium phosphate (pH 7.6) and 55% $(NH_4)_2SO_4$. Before each experiment an aliquot of the enzyme was collected by centrifugation, dissolved in 25 m*M* Tris (pH 8.0), 0.25 m*M* EDTA, and 10 m*M* $MgCl_2$, and applied to a small (1 × 10 cm) Sephadex G-25 column. The enzyme was eluted from the column with the same buffer, and collected in the first 2 ml after the void volume. The enzyme was then stored on ice for 4 hr to allow it to gain full activity (Bowes and Ogren, 1972; Pon et al., 1963). Ribulose 1,5-di-P carboxylase from leaves of spinach (*Spinacia oleracea* L.) was prepared and treated similarly.

Enzyme Assays. Purified ribulose 1,5-di-P carboxylase activity was assayed at 30° as described by Bowes and Ogren (1972) unless otherwise indicated. Reaction mixtures are specified in the table and figure legends. Reactions were initiated with enzyme, unless otherwise indicated, and terminated by the addition of 0.1 *N* HCl (final concentration). Samples were oven-dried at 60° and dissolved in 0.1 ml of water, and dpm was determined as described by Anderson and McClure (1973). Activity in crude extracts was measured as described by Bowes et al. (1972), except that the initial concentration of ribulose 1,5-di-P was 0.4 m*M* and 5 m*M* dithiothreitol replaced GSH. Although

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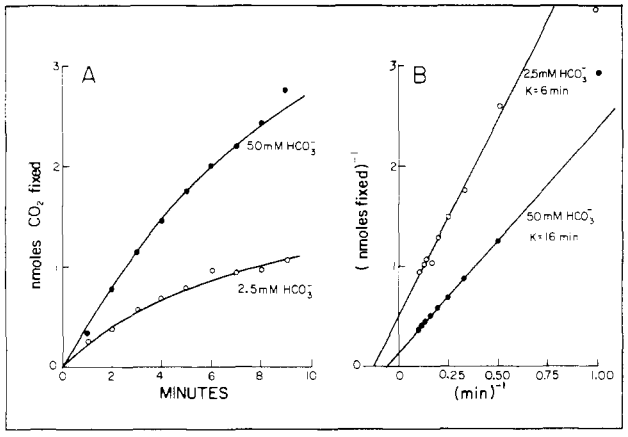

FIGURE 1: (A) Time courses of purified soybean ribulose 1,5-di-P carboxylase activity at 2.5 and 50 mM $NaHCO_3$. Reaction mixtures contained 50 mM Tris (pH 8.5), 0.4 mM ribulose 1,5-di-P, 6 mM $MgCl_2$, 0.1 mM EDTA, 60 μg of protein, and either 2.5 or 50 mM $NaH^{14}CO_3$ in a final volume of 3.0 ml. Reactions were initiated with protein, and 0.20-ml aliquots were removed and reactions terminated at the specified times. (B) Double reciprocal plots of the data in A.

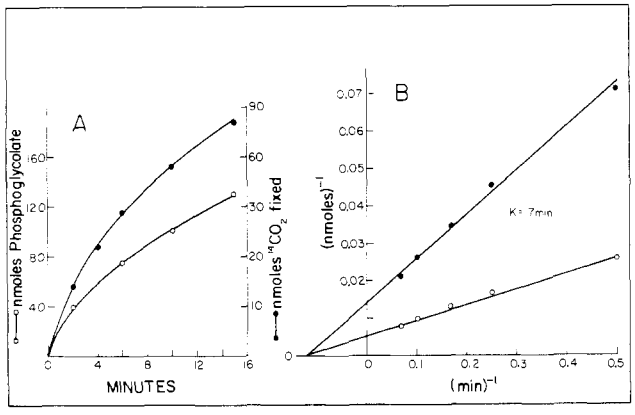

FIGURE 2: (A) Time courses of purified soybean ribulose 1,5-di-P carboxylase and oxygenase activities determined simultaneously in the same reaction flask at pH 8.5, 1.0 mM $NaHCO_3$ and 100% O_2. Reaction conditions as described under Materials and Methods. Each reaction flask contained 166 μg of protein. (B) Double reciprocal plots of the data in A.

CO_2, and not bicarbonate, is the substrate for ribulose 1,5-di-P carboxylase (Cooper et al., 1969) most results are expressed in terms of bicarbonate concentration because of the uncertainty in conversion constants relating CO_2 concentration to bicarbonate concentration. When CO_2 concentrations were calculated, the constants determined by Gibbons and Edsall (1963) were used.

Ribulose 1,5-di-P oxygenase activity was assayed by a modification of the colorimetric assay previously reported (Bowes et al., 1971). Ribulose 1,5-di-P oxygenase reaction mixtures contained 50 mM Tris (pH 8.5), 6 mM $MgCl_2$, 0.1 mM EDTA, 0.4 mM ribulose 1,5-di-P, and 75–200 μg of enzyme/ml. The P-glycolate produced by ribulose 1,5-di-P oxygenase activity was enzymatically converted to glyoxylate in the presence of P-glycolate phosphatase, glycolate oxidase, and catalase. Then glyoxylate phenylhydrazone was formed and converted to the 1,5-diphenylformazan derivative as described by Vogels and van der Drift (1970). The primary advantage of determining the concentration of the diphenylformazan derivative, compared to the phenylhydrazone derivative determined earlier (Bowes et al., 1971), is that the absorption maximum is shifted to 535 nm from 324 nm, greatly reducing background absorption. Full details of this procedure are available elsewhere (Laing, 1974).

In experiments where ribulose 1,5-di-P carboxylase and oxygenase activities were assayed simultaneously, the standard assays were slightly modified. After the flasks were flushed with gas and closed off, 1.0 mM $NaH^{14}CO_3$ was injected into the reaction flasks, followed by the addition of 0.6 mM (final concentration) ribulose 1,5-di-P and then enzyme. After the reactions were terminated with HCl, the flasks were flushed with N_2 for 5 min to remove the unreacted CO_2 and bicarbonate. Following enzymatic conversion of P-glycolate to glyoxylate, a 1.0-ml aliquot was removed for glyoxylate determination and an additional 0.5-ml aliquot was removed to a scintillation vial, dried, and dpm determined. In all experiments, the ribulose 1,5-di-P concentration at the end of the assay was greater than 0.4 mM, the saturation level for this substrate (Bowes and Ogren, 1972).

Protein concentrations were determined by the method of Warburg and Christian (1942).

Results

Time Course of Ribulose 1,5-Di-P Carboxylase Activity. Nanomoles of CO_2 incorporated by purified soybean ribulose 1,5-di-P carboxylase as a function of time at 2.5 and 50 mM $NaHCO_3$ (pH 8.5) is shown in Figure 1A. The time courses approximated hyperbolas, as shown in the double reciprocal plots in Figure 1B. Thus the time courses can be approximated by

$$n = Nt/(K + t) \quad (1)$$

where n in the nmoles of CO_2 incorporated at time t, N is the nmoles of CO_2 incorporated at infinite time (assuming that the hyperbola extends to infinite time), t is the time in minutes after initiation of assay, and K is a constant representing the half-time (in min) of initial enzyme activity. The slow, time-dependent change in activity characterizes ribulose 1,5-di-P carboxylase as a hysteretic enzyme (Frieden, 1970; Chu and Bassham, 1973). The data in Figure 1 suggest that high bicarbonate concentrations increase both the rate of reaction and the stability of ribulose 1,5-di-P carboxylase activity.

Time Courses of Ribulose 1,5-Di-P Carboxylase and Oxygenase Activities. When time courses of the carboxylation and oxygenation reactions catalyzed by ribulose 1,5-di-P carboxylase were examined simultaneously, nmoles of CO_2 incorporated and nmoles of P-glycolate produced declined with time (Figure 2A). The time courses of both reactions were hyperbolic and K was about 7 min for both reactions (Figure 2B). The decline in activity with time was not because of loss of substrate or end product inhibition, since the ribulose 1,5-di-P concentration was greater than saturation (0.4 mM; Bowes and Ogren, 1972) for the duration of the assay period and the total concentration of products at the end of the assay was less than 0.4 mM. The inhibition constants of the products of the reactions, 3-P-glycerate and P-glycolate, are 10 mM (Paulsen and Lane, 1966) and 3 mM (W. A. Laing, W. L. Ogren, and R. H. Hageman, unpublished), respectively, with respect to the carboxylation reaction. A hyperbolic time course for the ribulose 1,5-di-P oxygenase reaction was also found when this activity was assayed by measuring O_2 uptake with an O_2 electrode (data not shown).

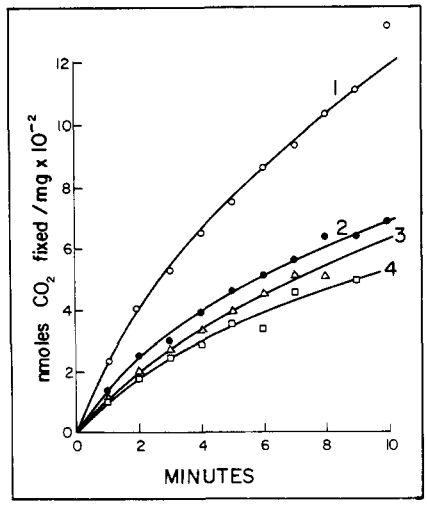

FIGURE 3: Time courses of purified soybean ribulose 1,5-di-P carboxylase activity at 5.0 m*M* $NaHCO_3$ at four O_2 concentrations. Reaction mixtures contained 50 m*M* Tris (pH 8.5), 5.0 m*M* $NaH^{14}CO_3$, 0.4 m*M* ribulose 1,5-di-P, 6 m*M* $MgCl_2$, 0.1 m*M* EDTA, and 48 μg of protein in a final volume of 1.0 ml. After flushing for 6 min with 21% O_2 (curve 1), 50% O_2 (curve 2), 75% O_2 (curve 3), or 100% O_2 (curve 4), the reaction flasks were sealed, followed by addition of $NaH^{14}CO_3$. The reactions were initiated with protein and terminated at the specified times with 0.1 *N* HCl.

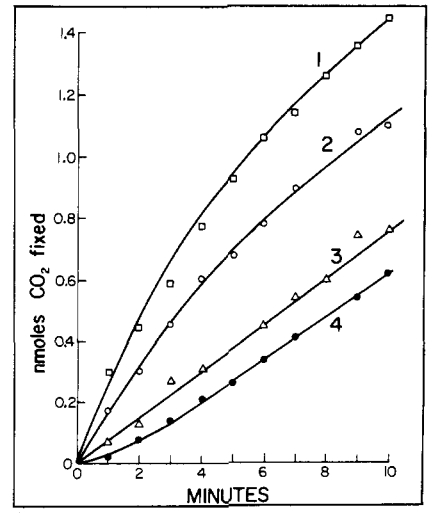

FIGURE 4: Effect of various preincubation treatments on the time course of purified soybean ribulose 1,5-di-P carboxylase activity. For all treatments, reaction mixtures were identical upon initiation of the reaction, and contained 50 m*M* Tris, (pH 8.0), 5.0 m*M* $NaH^{14}CO_3$, 0.4 m*M* ribulose 1,5-di-P, 6 m*M* $MgCl_2$, 0.1 m*M* EDTA, and 120 μg of protein in a final volume of 3.0 ml. After initiation of the reactions, 0.20-ml aliquots were removed and the reactions terminated at the specified times. (1) Standard assay, reaction was initiated with enzyme. (2) Enzyme was preincubated for 10 min at 30° in 10 m*M* Tris (pH 8.0), 25 m*M* $NaHCO_3$, and 5 m*M* $MgCl_2$. Reaction was initiated by addition of the remaining reaction components. (3) Enzyme was preincubated for 10 min at 30° in 20 m*M* Tris (pH 8.0) and 10 m*M* $MgCl_2$. Reaction was initiated by addition of the remaining reaction components. (4) Enzyme was preincubated for 10 min at 30° in 20 m*M* Tris (pH 8.0), 10 m*M* $MgCl_2$, and 2.0 m*M* ribulose 1,5-di-P. Reaction was initiated by addition of the remaining reaction components.

Effect of O_2 and Sulfhydryl-Protecting Agents on Ribulose 1,5-Di-P Carboxylase and Oxygenase Activities. To determine whether the observed decline in activities with time might be caused by oxidation of sulfhydryl groups on the enzyme, time courses of ribulose 1,5-di-P carboxylase and oxygenase activities were conducted under various O_2 tensions and in the presence and absence of sulfhydryl-protecting agents. The rate of decline in carboxylase activity with time, as established by determining *K*, was not influenced by O_2 tensions between 21 and 100% O_2 (Figure 3). In all cases, *K* was about 10 min. In another experiment, ribulose 1,5-di-P carboxylase activity was assayed under N_2, 2% O_2, and 21% O_2 (data not shown). Again, *K* was independent of O_2 tension. O_2 did inhibit ribulose 1,5-di-P carboxylase activity (Figure 3), as previously reported (Ogren and Bowes, 1971; Bowes and Ogren, 1972). The $K_i(O)_2$, determined from the data in Figure 3, was about 0.55 m*M* O_2 (50% O_2) at all assay times.

The ratio of ribulose 1,5-di-P carboxylase activity to ribulose 1,5-di-P oxygenase activity was not greatly affected by the addition of sulfhydryl-protecting agents when both activities were assayed simultaneously. Under conditions of 1.0 m*M* $NaHCO_3$ and 100% O_2, at pH 8.5, the ratio of carboxylase to oxygenase activity was approximately 0.37 throughout the 10-min assay period. The addition of 5 m*M* dithiothreitol had no effect on this ratio, but a ratio of 0.44 was observed in the presence of 5 m*M* GSH. As previously reported by this laboratory (Bowes and Ogren, 1972), 5 m*M* GSH reduces the O_2 concentration in solution under the assay conditions employed and this reduction in O_2 concentration might account for the observed increase in ratio. Dithiothreitol and GSH did not affect the stability of the enzyme with respect to either activity. Mercaptoethanol, cysteine, and 2,3-dimercaptopropanol did not affect *K* with respect to the carboxylase reaction. These compounds were not tested in the oxygenase reaction. The lack of significant effect of O_2 or sulfhydryl-protecting agents on the stability on the enzyme or on the ratio of the two activities suggests that oxidation of sulfhydryl groups of the enzyme is not causing the decline in activity with time.

The Effect of Preincubation and $MgCl_2$ Concentration on the Stability of Enzyme Activity. In the standard assay procedure employed in the experiments reported here, initiation of the reaction with enzyme, a hyperbolic time course was obtained at 5 m*M* $NaHCO_3$ (Figure 4, curve 1), with *K* equal to 10 min. When the enzyme was preincubated for 10 min at 30° in 25 m*M* $NaHCO_3$, 10 m*M* Tris (pH 8.0), and 5 m*M* $MgCl_2$, and the reaction initiated by addition of ribulose 1,5-di-P, a hyperbolic time course was again observed, with *K* equal to 10 min (Figure 4, curve 2). The initial rate of reaction was less than observed in the standard assay.

A linear time course was obtained, however, when the enzyme was preincubated for 10 min at 30° in 20 m*M* Tris (pH 8.0) and 5 m*M* $MgCl_2$ (no $NaHCO_3$), and the reaction was initiated by the addition of $NaHCO_3$ and ribulose 1,5-di-P (Figure 4, curve 3). Although the reaction rate was constant with time, the rate was only about one-third of the initial rate obtained with the standard assay. Incubation of the enzyme in 20 m*M* Tris (pH 8.0), 10 m*M* $MgCl_2$, and 2.0 m*M* ribulose 1,5-di-P for 10 min at 30°, followed by initiation of the reaction by $NaHCO_3$, caused a 3-min lag period, and then a constant rate for 7 min (Figure 4, curve 4). The final rate in this case was about the same as the rate observed following preincubation of the enzyme in Tris and $MgCl_2$. Upon initiation of the reactions illustrated in Figure 4, all reaction mixtures were identical. Thus it is possible to achieve linear time courses for ribulose 1,5-di-P carboxylase activity with appropriate preincubation, but the initial rates are greatly reduced. The time course of ribulose 1,5-di-P oxygenase activity was also linear for 4 min following prein-

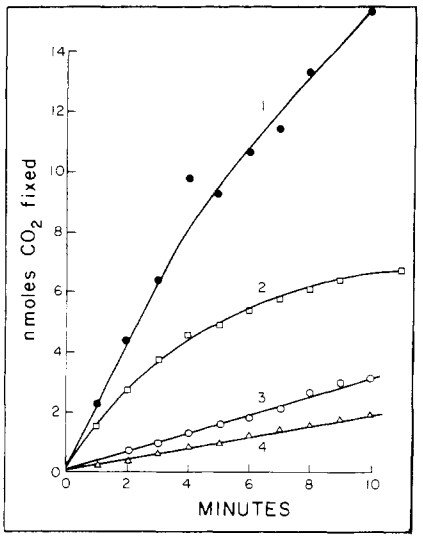

FIGURE 5: Time courses of purified soybean ribulose 1,5-di-P carboxylase activity at 2.5 and 50 mM $NaHCO_3$ in the presence of 6 and 46 mM $MgCl_2$. Reaction mixtures contained 50 mM Tris (pH 8.0), 2.5 or 50 mM $NaH^{14}CO_3$, 0.4 mM ribulose 1,5-di-P, 6 or 46 mM $MgCl_2$, 0.1 mM EDTA, and 330 μg of protein in a final volume of 3.0 ml. Reactions were initiated with protein, and 0.20-ml aliquots were removed and the reactions terminated at the specified times. (1) 50 mM $NaHCO_3$, 6 mM $MgCl_2$; (2) 2.5 mM $NaHCO_3$, 6 mM $MgCl_2$; (3) 50 mM $NaHCO_3$, 46 mM $MgCl_2$; (4) 2.5 mM $NaHCO_3$, 46 mM $MgCl_2$.

cubation of the enzyme in 50 mM Tris (pH 8.5), 6 mM $MgCl_2$, and 0.1 mM EDTA for 10 min at 30° (data not shown).

The decline in ribulose 1,5-di-P carboxylase activity with time could also be overcome at low $NaHCO_3$ concentration by substantially increasing the $MgCl_2$ concentration in the reaction mixture. At 50 mM $NaHCO_3$ and 6 mM $MgCl_2$, the reaction rate was initially constant (Figure 5, curve 1). At 2.5 mM $NaHCO_3$ and 6 mM $MgCl_2$, a hyperbolic time course was observed, with K equal to 7 min (Figure 5, curve 2). In the presence of 46 mM $MgCl_2$, the reaction rates were constant with time at both 50 mM $NaHCO_3$ (Figure 5, curve 3) and 2.5 mM $NaHCO_3$ (Figure 5, curve 4). As reported by Sugiyama et al. (1968a,b), $MgCl_2$ at high concentrations is strongly inhibitory to ribulose 1,5-di-P carboxylase activity under alkaline conditions.

The Effect of $NaHCO_3$ Concentration on the Stability of Ribulose 1,5-Di-P Carboxylase Activity. The effect of $NaHCO_3$ concentration on enzyme activity during assay was determined by conducting time courses of ribulose 1,5-di-P carboxylase activity at five different $NaHCO_3$ concentrations. Double reciprocal plots of these time courses were constructed and the values for K (enzyme activity half-time) and N (nmoles CO_2 incorporated at infinite time) determined. These values are listed in Table I. The constant K increased from 6 min at 2.5 mM $NaHCO_3$ to about 17 min at 25 mM $NaHCO_3$.

Since the decline in carboxylase activity is less at higher $NaHCO_3$ concentrations, kinetic constants (e.g., K_m, V) determined for ribulose 1,5-di-P carboxylase with respect to CO_2 will be incorrect. At lower $NaHCO_3$ concentrations, the rate of carboxylase activity determined during any finite time period will be less than expected on the basis on standard enzyme kinetics, thereby causing an increase in the apparent K_m value and a decrease in the apparent V. The effect on apparent K_m is illustrated in Figure 6. From the values of K and N in Table I, the nmoles of CO_2 incorporated by ribulose 1,5-di-P at times t (t equals 0, 2, 4, 6, 8, and 10 min) were calculated from

Table I: Effect of Bicarbonate Concentration on K and N.[a]

$NaHCO_3$ (mM)	K (min)	N (nmol)
2.5	6.0	17
3.3	7.7	24
5.0	9.5	33
10	14.9	56
25	16.7	72

[a] K and N (defined in eq 1) were determined from time courses of purified soybean ribulose 1, 5-di-P carboxylase activity at the specified $NaHCO_3$ concentrations. Reaction mixtures are described in the legend to Figure 5.

$$v_t = NK/(K + t)^2 \quad (2)$$

From the calculated v_t values, Lineweaver-Burk plots ($1/v$ vs. $1/S$) were constructed for each of the six times (Figure 6). The apparent K_m increased from 1.3 mM $NaHCO_3$ at zero time to 17 mM $NaHCO_3$ at 10 min (Figure 6, inset). Thus the apparent K_m determined for ribulose 1,5-di-P carboxylase with respect to CO_2 (or $NaHCO_3$), if the reactions are initiated with enzyme, depends on the length of the assay period. The apparent K_m calculated at zero time in Figure 6 (inset) is similar to that observed in intact chloroplasts (Jensen and Bassham, 1966), leaf cells (Jensen et al., 1971), and leaves (Goldsworthy, 1968; Akita and Moss, 1973), while the apparent K_m calculated at 5 min and longer times is similar to that reported for the isolated carboxylase (Ogren and Bowes, 1971; Bowes and Ogren, 1972; Paulsen and Lane, 1966). As will be discussed, we interpret the apparent change in K_m with time to be a result of changes in V of ribulose 1,5-di-P carboxylase.

The Effect of O_2 on the Stability of Ribulose 1,5-Di-P Oxygenase Activity. Since ribulose 1,5-di-P carboxylase activity was found to be stabilized by high $NaHCO_3$ concentrations (Table I), one might expect, by analogy, a similar response by the enzyme to its alternate substrate, O_2 (Bowes et al., 1971). Ribulose 1,5-di-P oxygenase activity was measured as a function of time at 0.21, 1.0, and 5 atm of O_2 (Figure 7). An identical decline in activity was found under all conditions, with K equal to 6 min. Thus O_2 does not stabilize the enzyme as does $NaHCO_3$. The data in Figure 7 provide additional evidence that O_2 does not cause the time-dependent decline in enzyme activity.

Discussion

If the carboxylase and oxygenase activities of ribulose 1,5-di-P carboxylase are described by standard enzyme kinetics, then

$$v_c = V_c K_o C/(K_c K_o + K_c O + K_o C) \quad (3)$$

and

$$v_o = V_o K_c O/(K_c K_o + K_c O + K_o C) \quad (4)$$

where v is the velocity of the reaction, subscript "c" refers to CO_2, subscript "o" refers to O_2, V is the maximal velocity, K_c and K_o are the Michaelis (or inhibition) constants, C is the CO_2 concentration, and O is the O_2 concentration. The use of the terms K_c, for $K_m(CO_2)$ in the carboxylase reaction and $K_i(CO_2)$ in the oxygenase reaction, and K_o, for $K_m(O_2)$ in the oxygenase reaction and $K_i(O_2)$ in the carboxylase reaction are justified since, for soybean ribulose

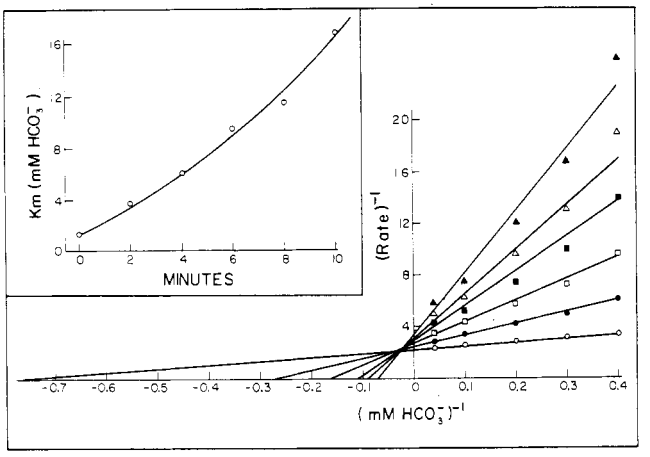

FIGURE 6: Double reciprocal plot of rate of purified soybean ribulose 1,5-di-P carboxylase vs. bicarbonate concentration at different times. Inset: Apparent K_m as a function of time. Reaction mixtures contained 50 mM Tris (pH 8.5), 2.5, 3.3, 5.0, 10, or 25 mM $NaH^{14}CO_3$, 0.4 mM ribulose 1,5-di-P, 6 mM $MgCl_2$, 0.1 mM EDTA, and 140 μg of protein in a final volume of 3.0 ml. Reactions were initiated with protein and 0.20-ml aliquots removed at times 2, 4, 6, 8, and 10 min. Further details as described in Results.

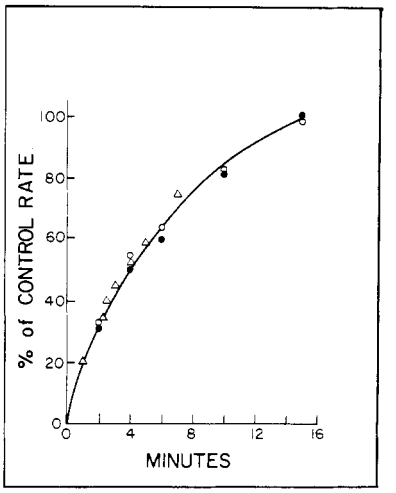

FIGURE 7: Time courses of purified soybean ribulose 1,5-di-P oxygenase activity at 0.21, 1.0 and 5 atm of O_2. Reactions were conducted as described under Materials and Methods. The control rate for each O_2 tension was normalized to the amount of P-glycolate produced after 15 min of reaction.

1,5-di-P carboxylase, $K_m(CO_2)$ in the carboxylase reaction equals $K_i(CO_2)$ in the oxygenase reaction, and $R_m(O_2)$ in the oxygenase reaction equals $K_i(O_2)$ in the carboxylase reaction (Laing et al., 1974). From eq 3 and 4, the ratio of carboxylase activity to oxygenase activity under identical reaction conditions can be described (eq 5). Therefore, at constant C and O under identical reaction conditions, the ratio v_c/v_o should be constant.

$$v_c/v_o = V_cK_oC/V_oK_cO \quad (5)$$

The decline in v_c and v_o with time was equal for both the carboxylase and oxygenase activities of ribulose 1,5-di-P carboxylase at 1.0 mM $NaHCO_3$ and 100% O_2, and the ratio of the two activities remained constant with time (Figure 2). Figure 6 suggests that K_c [$K_m(CO_2)$] increases with time of assay, and previous investigators have concluded that K_c of the carboxylase increases upon removal of the enzyme from the chloroplast (Jensen, 1971; Walker, 1971, 1973; Bahr and Jensen, 1974). If K_c is increasing, then, from eq 5, either K_o, V_c, or V_o must also be changing to account for the observation that v_c/v_o is constant with time. The available evidence indicates that K_o does not change. The $K_i(O_2)$ for purified soybean ribulose 1,5-di-P carboxylase (Ogren and Bowes, 1971; Bowes and Ogren, 1972) and the inhibition constant for O_2 on carboxylation efficiency in soybean leaf photosynthesis (Ogren and Bowes, 1971; Forrester et al., 1966) are approximately equal, the $K_m(O_2)$ in the oxygenase assay (Laing et al., 1974; Andrews et al., 1973) approximates the $K_m(O_2)$ in soybean leaf photorespiration (calculated from Figure 8 in Forrester et al., 1966), and the $K_i(O_2)$ in the carboxylase assay at low bicarbonate concentrations is constant with time (calculated from the data in Figure 3). All measurements of K_o, determined as either $K_m(O_2)$ in the oxygenase reaction of $K_i(O_2)$ in the carboxylase reaction, are in the range of 40–70% O_2.

V_c cannot be increasing to compensate for an increase in K_c, for in this case there would be no decline in oxygenase activity with time. Oxygenase activity declined in a manner identical with the decline in carboxylase activity (Figure 2). There is no evidence to indicate that identical, reciprocal changes could not be occurring in K_c and V_o, but we consider it unlikely that these two particular kinetic constants would be so closely linked.

The data presented here can be best explained on the basis that the enzyme changes which are occurring affect V, that is, affect V_c and V_o identically. Upon initiation of reaction by addition of enzyme to a reaction mixture, the enzyme, initially in a high-activity form designated E_1, undergoes hysteretic conversion to a second, less active or inactive, form E_2. E_2 is rapidly activated to E_1 by bicarbonate, Mg^{2+}, and possibly certain sugar phosphates (to be discussed later). The activation of E_2 to E_1 is bicarbonate-concentration dependent. The constant K (eq 1) indicates the net conversion of E_1 to E_2, and is thus a function of both the decay of E_1 to E_2 and the activation of E_2 to E_1. At high (50 mM) $NaHCO_3$, the equilibrium greatly favors the activation of E_2 to E_1, so when E_1 decays to E_2, E_2 is rapidly activated back to E_1, and activity declines only slowly with time. In the absence of $NaHCO_3$ (e.g., an assay of only ribulose 1,5-di-P oxygenase activity), no activation of E_2 to E_1 occurs, and the reaction rate rapidly declines with time. At intermediate $NaHCO_3$ concentrations, E_1 decays to E_2, and some E_2 is activated to E_1, the extent of activation increasing at higher $NaHCO_3$ concentrations. Thus the measured constant K increases as the $NaHCO_3$ concentration increases. These observations are consistent with the suggestion of Chu and Bassham (1973) that ribulose 1,5-di-P binding at CO_2 activating sites inactivates the enzyme, and that high levels of bicarbonate overcome inactivation by displacing the bound ribulose 1,5-di-P.

The slow decay of enzyme from E_1 to E_2 and the bicarbonate-dependent activation of E_2 to E_1 can explain the high apparent $K_m(CO_2)$ values previously reported for purified ribulose 1,5-di-P carboxylase when activity is assayed by initiation of the reactions by enzyme (Ogren and Bowes, 1971; Bowes and Ogren, 1972; Paulsen and Lane, 1966). At saturating $NaHCO_3$ concentration, the maximal rate is observed. As the $NaHCO_3$ concentration is lowered, the rate of reaction is reduced not only because less substrate is available, but also because more enzyme is present in a less active, or inactive, form. Since the rate of reaction at lower $NaHCO_3$ concentration is less than would be observed with an enzyme following standard linear kinetics, the velocity of

the reaction at lower $NaHCO_3$ concentration will be less than expected, and conventional Lineweaver-Burk plots ($1/v$ vs. $1/S$) will show an increased slope and an increase in apparent K_m. The complications of enzyme decay and activation can be overcome if $K_m(CO_2)$ determinations are based on initial rates, as shown in Figure 6.

The hypothesis suggested above predicts that ribulose 1,5-di-P carboxylase will be predominantly in form E_2 following preincubation of the enzyme in the absence of $NaHCO_3$. Initiation of the reaction with $NaHCO_3$ rapidly converts E_2 to E_1, so reaction rates are constant with time (Figure 4, curve 3). Since the extent of conversion of E_2 to E_1 is a function of the bicarbonate concentration, bicarbonate should exert an allosteric effect on the enzyme when the enzyme is assayed after preincubation. Although we did not look for this effect, data from three laboratories suggest that bicarbonate (or CO_2) exerts a homotrophic effect on purified ribulose 1,5-di-P carboxylase (Sugiyama et al., 1968b; Andrews and Hatch, 1971; Murai and Akazawa, 1972; Buchanan and Schürmann, 1973). In these reports, activity was assayed after preincubation of the enzyme. We are not aware of any experiments where cooperativity with bicarbonate was obtained when the assays were initiated with enzyme.

The apparent $K_m(CO_2)$ for ribulose 1,5-di-P carboxylase was reduced by assaying the enzyme in the presence of high $MgCl_2$ concentrations (Sugiyama et al., 1968a,b; Bassham et al., 1968) or in the presence of certain sugar phosphates (Buchanan and Schürmann, 1973). These compounds may also affect the equilibrium between E_1 and E_2, either by preventing decay of E_1 to E_2 or by stimulating the conversion of E_2 to E_1. As shown in Figure 5, the time course of ribulose 1,5-di-P carboxylase activity was linear at 2.5 m*M* $NaHCO_3$ in the presence of 46 m*M* $MgCl_2$, but curvilinear at 6 m*M* $MgCl_2$. The data in Figure 5 permit an estimation of $K_m(CO_2)$ at the two $MgCl_2$ concentrations. In 6 m*M* $MgCl_2$, the apparent K_m increased from 0.9 m*M* $NaHCO_3$ at zero time to 9 m*M* $NaHCO_3$ after 10 min. In 46 m*M* $MgCl_2$, the apparent K_m was 1.7 m*M* $NaHCO_3$ at all times.

Buchanan and Schürmann (1973) reported that the $K_m(CO_2)$ for ribulose 1,5-di-P carboxylase was considerably reduced when the enzyme was assayed in the presence of either fructose-6-P or 6-P-gluconate. In their experiments (Buchanan and Schürmann, 1973), carboxylase activity was assayed after preincubation. When the enzyme was assayed in the absence of the sugar phosphates, sigmoidal kinetics and a high apparent $K_m(CO_2)$ were observed. When either fructose-6-P or 6-P-gluconate was added to the reaction mixture, the apparent $K_m(CO_2)$ was reduced and linear kinetics were observed. These observations are consistent with the suggestion that these two sugar phosphates also maintain ribulose 1,5-di-P carboxylase in form E_1, as do high concentrations of $MgCl_2$ and $NaHCO_3$.

The decay of ribulose 1,5-di-P carboxylase from E_1 to E_2 and the activation of E_2 to E_1 by bicarbonate may be important in the light activation of photosynthesis. CO_2 fixation in photosynthetic organisms ceases in the dark, even though both CO_2 and ribulose 1,5-di-P are available (Pedersen et al., 1966). On illumination of a leaf or algal cell, protons are pumped from the chloroplast stroma, the site of ribulose 1,5-di-P carboxylase, into the grana (Packer et al., 1970), so the pH in the vicinity of the carboxylase increases. Since the solubility of CO_2 is not greatly affected by pH, an increase in pH in the chloroplast stroma will cause the bicarbonate concentration to increase. An increase in bicarbonate concentration could activate the carboxylase from E_2 to E_1, allowing CO_2 fixation to begin. An 18-fold increase in chloroplast bicarbonate concentration has been observed following illumination (Werdan and Heldt, 1972). Following the onset of darkness, the pH in the stroma decreases, causing the bicarbonate concentration to decrease. As the bicarbonate concentration decreases, E_1 could decay to E_2.

Activation of ribulose 1,5-di-P carboxylase in vivo has previously been attributed to light-induced increases in the concentrations of Mg^{2+} (Sugiyama et al., 1968a,b; Bassham et al., 1968) and fructose-6-P (Buchanan and Schürmann, 1973) at the site of the carboxylase. Bicarbonate, Mg^{2+}, and fructose-6-P may act separately or together to activate the carboxylase to a more active form in the light. The data presented in this paper suggest that the activation of ribulose 1,5-di-P carboxylase consists of inducing a modification of the enzyme from a form with a lower maximal velocity to a form with a higher maximal velocity, and that the activation does not involve changes in the $K_m(CO_2)$.

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Zinc and Magnesium Content of Alkaline Phosphatase from *Escherichia coli*†

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ABSTRACT: Since alkaline phosphatase from *Escherichia coli* was first reported to contain 2.1 g-atoms of zinc and 0.8 g-atom of magnesium per molecular weight 80,000 (Plocke, D. J., Levinthal, C., and Vallee, B. L. (1962), *Biochemistry 1,* 373–378), the procedures for isolation and purification of the enzyme, as well as values for the protein molecular weight, specific absorptivity, and maximal activity, have changed repeatedly. Such variations have resulted in uncertainties concerning the molar metal content of this phosphatase. The present paper reviews the initial and recent results of metal analyses of alkaline phosphatase preparations in this laboratory and compares them with those obtained elsewhere, while simultaneously identifying some of the factors which have affected reports on the metal content of this enzyme. A purification procedure is described eliminating the features of all methods known to alter the metal content of phosphatase. In addition, the three isozymic forms, as well as preparations from four *E. coli* strains commonly employed for phosphatase isolation, were analyzed and compared. Collectively, the zinc content was found to be 4.0 ± 0.3 g-atoms per molecular weight of 89,000 for enzyme purified and analyzed by procedures shown not to alter its intrinsic metal content adversely. Importantly, it is confirmed that the enzyme when isolated at pH 7.2 and 7.5 contains an average of 1.3 ± 0.2 g-atoms of magnesium/mol. This feature of the metal composition, hitherto largely unappreciated, bears significantly on the investigation of the structure of alkaline phosphatase as related to its chemical and functional properties.

The precision and sensitivity of methods applicable to the determination of the physical, chemical, and functional properties of enzymes have increased markedly in the last decade. Concurrently, there have been analogous advances in high resolution procedures for enzyme isolation. Purification of enzymes by means of such superior methodology may result in numerical values for the parameters characterizing their properties which differ from those obtained initially. Unfortunately, such determinations frequently do not indicate definitively whether the numerical changes observed result from intrinsic differences in the enzyme being examined, variations in analytical methodology, or differing modes of calculation and representation of results.

Escherichia coli alkaline phosphatase well illustrates these evolving problems in protein characterization. It was first isolated in 1960 (Garen and Levinthal, 1960) and soon thereafter shown to contain stoichiometric quantities of zinc and magnesium (Plocke et al., 1962). Since these early studies, native phosphatase, prepared from *E. coli* by a variety of methods, consistently has been shown to require minimally 2 g-atoms of zinc/mol of enzyme for catalytic activity (Simpson et al., 1968; Harris and Coleman, 1968; Reynolds and Schlesinger, 1969; Petitclerc et al., 1970; Trotman and Greenwood, 1971; Csopak and Szajn, 1973). Further, it was demonstrated that two additional zinc atoms stabilize secondary, tertiary, or quaternary structure and/or

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