EFFECTS OF CO_2, O_2 AND TEMPERATURE ON A HIGH-AFFINITY FORM OF RIBULOSE DIPHOSPHATE CARBOXYLASE-OXYGENASE FROM SPINACH

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SUMMARY: A high-affinity form of ribulose diphosphate carboxylase, observed transiently in spinach-leaf extracts soon after extraction, was inhibited by O_2 competitively with respect to CO_2. Analogously, the ribulose diphosphate oxygenase activity for this form was inhibited by CO_2, competitively with respect to O_2. For each gas, the K_m for the reaction in which it was a substrate was similar to its K_i for the reaction it inhibited. The Arrhenius activation energy for the oxygenase reaction was 1.5 times that of the carboxylase. These characteristics are consistent with ribulose diphosphate oxygenase being the enzymatic reaction responsible for synthesizing the substrate for photorespiration and with the concept that the balance between photosynthesis and photorespiration of leaves is a reflection of the ratio between the two activities of this bi-functional enzyme.

Following the observation that photorespiring spinach leaves rapidly incorporated $[^{18}O]O_2$ into the carboxyl groups of the photorespiratory intermediates, glycine and serine (1), it was demonstrated that phosphoglycolate, the phosphorylated form of the photorespiratory substrate, glycolate, arose by the direct oxygenation of RuDP*, the primary carboxyl acceptor of C_3 photosynthesis, according to the following equation (2,3,4).

$$\text{RuDP} + O_2 \rightarrow \text{phosphoglycolate} + \text{3-phosphoglycerate}$$

Thus the initial reactions of both photosynthesis and photorespiration compete for the same substrate, RuDP, and furthermore these two reactions appear to be catalysed by the same protein (3).

Until recently the properties of RuDP carboxylase-oxygenase *in vitro* were not consistent with those of photosynthesis and photorespiration *in vivo* in two important respects, namely: (i) the $K_m(CO_2)$ for the carboxylase reaction was at least an order of magnitude higher than that of intact leaves or isolated chloroplasts (5), and (ii) in view of its very alkaline pH optimum, the activity of the oxygenase reaction at physiological pH appeared to be inadequate to account for the observed rate of photorespiration (3). The first of these discrepancies was resolved by the recent demonstration of a

*Abbreviations:RuDP, D-ribulose-1,5-diphosphate; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; Bicine, N,N-bis(2-hydroxyethyl)glycine; ammediol, 2-amino-2-methyl-1,3-propandiol.

form of RuDP carboxylase obtained from ruptured spinach chloroplasts and maize leaves, and stablized by Mg^{2+}, ATP and ribose-5-phosphate, which has a $K_m(CO_2)$ not much greater than that displayed by intact leaves (6,7). We have been able to confirm these observations and to show that the pH *vs* activity profiles of both carboxylase and oxygenase activities of this form are quite broad, both with optima in the region pH 8.2-8.6 (8), thus resolving the second discrepancy.

Gas-exchange studies with leaves of C_3 plants consistently show that photosynthesis is stimulated by increasing CO_2 concentrations and inhibited by increasing O_2 concentrations, with the reverse applying for photorespiration. Additionally, increasing the temperature favours photorespiration at the expense of photosynthesis (9,10). It is our aim in this paper to show that these phenomena may be explained in terms of the effects of CO_2,O_2 and temperature on RuDP carboxylase-oxygenase.

METHODS: Mature leaves of greenhouse-grown spinach (*Spinacia oleracia* hybrid 102) were pre-illuminated (230 W. m^{-2}) for at least 30 min and then ground with sand in a mortar in two volumes of a buffer solution containing 100 mM HEPES*-NaOH, pH 7.8, 25 mM $MgCl_2$, 5 mM dithiothreitol, 2 mM ribose-5-phosphate (Sigma Chemical Co.) and 3 mM ATP. The homogenate was filtered through Miracloth (Chicopee Mills, Inc.) and a sample of the filtrate taken for chlorophyll determination (11). The remainder of the filtrate was centrifuged (25,000 x g, 5 min) and a sample of the supernatant freed of low-molecular-weight compounds by passage through a small column of Sephadex G-25, equilibrated with 20 mM HEPES-NaOH, pH 8.3, buffer solution, containing 25 mM $MgCl_2$, 0.5 mM dithiothreitol, 2 mM ribose-5-phosphate and 3 mM ATP. These extraction procedures were carried out at 2° and were completed in about 30 min. The extract could be stored for a further 90 min at 0° without appreciable loss of activity or increase in $K_m(CO_2)$ (8).

RuDP carboxylase was assayed by measuring fixation of $^{14}CO_2$ into acid-stable products at 25° in a solution containing 100 mM Bicine*- or HEPES-NaOH, pH 8.3 (unless otherwise stated), 13 mM $MgCl_2$, 5 mM dithiothreitol and 0.8 mM RuDP (Sigma Chemical Co.). Total volume was 0.4 ml and the assay was performed in 5 ml vials with serum stoppers. The required gas phase, obtained by mixing humidified O_2 and N_2 with accurate gas-mixing pumps (H. Wösthoff, Bochum), was bubbled through the solution for 10 min by means of capillary tubes inserted through the stoppers. The capillaries were then removed, the required amount of $NaH^{14}CO_3$ (0.2μC/μmole) injected, and the reaction started by the injection of enzyme extract. Samples (100μl) were taken after 30 and 60 sec and added to 1 ml of 10% formic acid in scintillation vials. After drying, fixed ^{14}C was determined by scintillation counting.

In accordance with previous observations (6), the linearity of the reaction deteriorated after the first minute.

RuDP oxygenase was assayed by measuring O_2 uptake at 25° with an O_2-electrode (Rank Bros, Bottisham, Cambridge). The assay solution contained 100 mM Bicine-NaOH or ammediol*-HCl, pH 8.35, 15 mM $MgCl_2$, 2 mM ATP and 1 mM ribose-5-phosphate. No differences were observed if 0.8 mM RuDP replaced ATP and ribose-5-phosphate. The required O_2 concentration was established by bubbling the solution with CO_2-free air, or mixtures of O_2 and N_2 obtained using the gas-mixing pumps. The electrode vessel was then stoppered and the required CO_2 concentration obtained by adding a calculated amount of $NaHCO_3$ (see below). The reaction was started by adding enzyme extract. Final volume was 2.5 ml and the final concentration of dithiothreitol carried over with the enzyme solution was 0.1 mM. Again, linearity quickly deteriorated and activity was measured during the first two minutes only. A small rate of O_2 uptake occurring the absence of substrate was subtracted. The electrode was calibrated with solutions saturated with known mixtures of O_2 and N_2, prepared with the gas-mixing pumps.

Endogenous CO_2 and bicarbonate were removed from all assay solutions before addition of RuDP by purging with N_2 or CO_2-free air at pH 4.0-4.5 for 10 min. The pH was then adjusted to the desired value with carbonate-free NaOH. Subsequent exposure of assay solutions to atmospheric CO_2 was avoided. The concentration of dissolved CO_2, the form of inorganic carbon used by RuDP carboxylase (12), was calculated from pH and bicarbonate concentration using the Henderson-Hasselbach equation with a value of 6.37 for the pK', at 25°, of the CO_2-hydration reaction (13).

RESULTS AND DISCUSSION: O_2 inhibited the low-$K_m(CO_2)$ form of RuDP carboxylase in a classically competitive manner with respect to CO_2 (Figure 1).

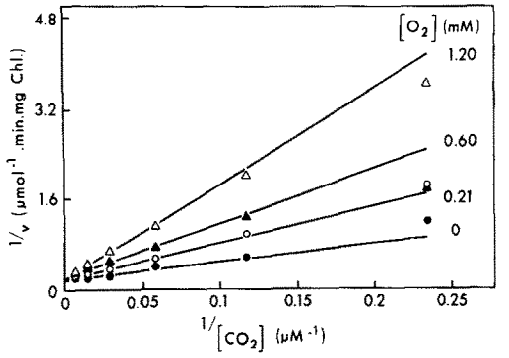

FIGURE 1. Double reciprocal plots of RuDP carboxylase activity as a function of CO_2 and O_2 concentrations. The pH of the assay solution was 8.05. Other details are given in the Methods section. The slopes and intercepts of the lines were calculated according to Wilkinson (20).

Except for the much lower $K_m(CO_2)$, these results are similar to those obtained by Bowes and Ogren (14). In an entirely analogous manner, CO_2 inhibited RuDP oxygenase activity competitively with respect to O_2 (Figure 2).

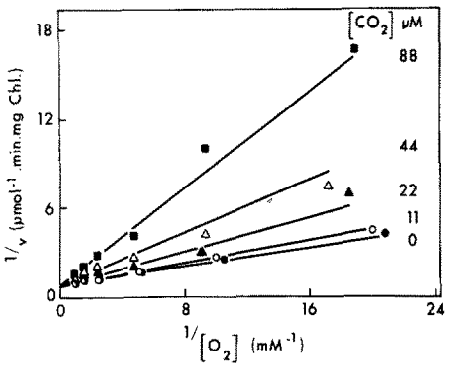

FIGURE 2. Double reciprocal plots of RuDP oxygenase activity as a function of O_2 and CO_2 concentrations. Details of the assays are given in the Methods section. The slopes and intercepts of the lines were calculated according to Wilkinson (20).

The kinetic constants for CO_2 and O_2 for both carboxylase and oxygenase, obtained from the experiments shown in Figures 1 and 2 and similar experiments at different pH or with different buffers, are summarized in Table 1. For each gas, the K_m for the reaction in which it was a substrate was similar to its K_i for the reaction in which it was an inhibitor. These observations support the contention that both reactions are catalysed by the same active site of the same protein (3). The $K_m(CO_2)$ of the carboxylase reaction was the same at both pH values tested although the bicarbonate concentration required to maintain this CO_2 concentration was proportionally higher at the higher pH. This $K_m(CO_2)$ is similar to that reported by Bahr and Jensen (6) and is comparable to that of CO_2 fixation by intact chloroplasts (15). The higher affinity of this form of the enzyme for CO_2 was accompanied by a higher affinity for O_2. Both the $K_m(O_2)$ for the oxygenase and the $K_i(O_2)$ for the carboxylase were substantially lower than observed previously (3,14). This strict competitivity between CO_2 and O_2 for both carboxylase and oxygenase reactions is analogous to the competitivity between CO_2 and O_2 observed in gas-exchange studies of photosynthesis and photorespiration in leaves of C_3 plants (9,10).

Using the means of the kinetic constants and maximum velocities given in Table 1 it is possible to calculate the levels of both activities which would be observed for any mixture of CO_2 and O_2. The equations described by Dixon and Webb (16) for an enzyme catalysing two reactions simultaneously may

TABLE 1. Kinetic properties of RuDP carboxylase-oxygenase.

Buffer	pH	K_m or K_i for CO_2 (μM)[2]	K_m or K_i for O_2 (μM)	V_{max}[1,3]	v_{air}[2,3]
		CARBOXYLASE			
Bicine	8.05	19	307	5.63	
Bicine	8.47	16	400	5.67	
MEAN:		17.5	354	5.65	1.43
		OXYGENASE			
Ammediol	8.35	18	208	1.14	
Bicine	8.35	21	183	1.30	
MEAN:		19.5	196	1.22	0.56

1. Maximum rates at saturating substrate concentrations.
2. Calculated rates at concentrations of CO_2 and O_2 in air-saturated solution at 25° (10.2 μM CO_2, 253 μM O_2).
3. $\mu mol.\ min^{-1}.\ mg\ chlorophyll^{-1}$.

be used. The calculated activities for the CO_2 and O_2 concentrations of an air-saturated solution (Table 1) predict an oxygenase:carboxylase activity ratio of 0.4 under "*in vivo*" conditions. Since both oxygenase and carboxylase activities of this high-affinity form of the enzyme have very broad and almost co-incident *pH vs* activity profiles (8), this ratio would not be very pH-dependent. For photorespiration to produce one molecule of CO_2, two molecules of glycolate must be metabolised via the glycolate pathway (17). Therefore, the calculated ratio implies that CO_2 should be released by photorespiration at 20% of the rate at which it is fixed by photosynthesis. This prediction agrees well with $^{14}CO_2$-exchange measurements of photorespiration in the presence of photosynthesis for a variety of C_3 plants (18,19). This correlation must be considered as approximate only, since the intracellular concentrations of O_2 and, more importantly, CO_2 in a leaf cannot be at complete equilibrium with atmospheric concentrations because of stomatal limitations to free gas exchange. However, these limitations do not apply at the CO_2-compensation point which is an equilibrium situation where the atmospheric CO_2 concentration must accurately reflect the intracellular concentration. Using the same set of equations (16) and the data in Table 1, it is possible to predict the CO_2-compensation concentration of spinach leaves. In accordance with the stoichiometry of the glycolate pathway (17), this will be

the CO_2 concentration at which oxygenation proceeds at twice the rate of carboxylation. At 21% O_2 and 25°, a value of 1.9 μM is obtained. This corresponds to an atmospheric CO_2 concentration of 60 p.p.m. which is close to the CO_2-compensation point exhibited by most C_3-plant leaves.

With C_3 plants, increasing temperature favours photorespiration at the expense of photosynthesis and consequently increases the CO_2-compensation point (9,10). While this effect may be partly due to the decreasing ratio between the solubilities of CO_2 and O_2 with increasing temperature (13), the present studies revealed another likely cause. Increasing temperature caused a much greater increase in RuDP oxygenase activity than in RuDP carboxylase activity. Calculations from Arrhenius plots (Figure 3) showed that the

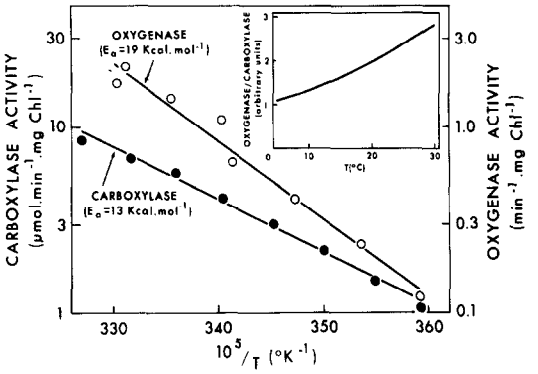

FIGURE 3. Effect of temperature on activities of RuDP carboxylase and RuDP oxygenase (Arrhenius plots). RuDP carboxylase was assayed at pH 8.2 (adjusted at each temperature) with 12.5 mM $NaH^{14}CO_3$. RuDP oxygenase was assayed at air levels of O_2 and the psuedo-first-order rate constant calculated as described in the text. INSET. Replot showing the effect of temperature on the ratio between RuDP oxygenase and RuDP carboxylase activities.

activation energy of the oxygenase reaction was 1.5 times that of the carboxylase. It was essential in this experiment to ensure that no part of this difference was attributable to the solubilities of CO_2 and O_2 changing with temperature. In the case of the carboxylase this was done by supplying a constant and saturating level of CO_2 plus bicarbonate at all temperatures. Since oxygenase activity was barely saturated even at 100% O_2, a similar procedure could not be adopted in this case. Instead, a pseudo-first-order rate constant for oxygenase activity was calculated at each temperature by assuming that activity was approximately linearly dependent on O_2 concentration at concentrations around the $K_m(O_2)$ or below and dividing the observed rate by the O_2 concentration at which it was observed. The

resultant value is dimensionless as far as O_2 is concerned. Unfortunately, this means that the ratio between oxygenase and carboxylase activities at various temperatures can be calculated in arbitrary units only (Figure 3, inset). However, there is a 2.5-fold increase in this ratio between 5° and 30° and this could probably entirely account for the temperature dependence of the CO_2-compensation point.

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REFERENCES:

1. Andrews, T.J., Lorimer, G.H., and Tolbert, N.E. (1971) Biochemistry 10, 4777-4782.
2. Bowes, G., Ogren, W.L., and Hageman, R.H. (1971) Biochem. Biophys. Res. Commun. 45, 716-722.
3. Andrews, T.J., Lorimer, G.H., and Tolbert, N.E. (1973) Biochemistry 12, 11-18.
4. Lorimer, G.H., Andrews, T.J., and Tolbert, N.E. (1973) Biochemistry 12, 18-23.
5. Walker, D.A. (1973) New Phytol. 72, 209-235.
6. Bahr, J.T., and Jensen, R.G. (1974) Plant Physiol. 53, 39-44.
7. Bahr, J.T., and Jensen, R.G. (1974) Biochem. Biophys. Res. Commun. 57, 1180-1185.
8. Badger, M.R., Stewart, A., and Andrews, T.J. Manuscript in preparation.
9. Jackson, W.A., and Volk, R.J. (1970) Ann. Rev. Plant Physiol. 21, 385-432.
10. Jollife, P.A., and Tregunna, E.B. (1973) Can. J. Bot. 51, 841-853.
11. Arnon, D.I. (1949) Plant Physiol. 24, 1-15.
12. Cooper, T.G., Filmer, D., Wishnick, M., and Lane, M.D. (1969) J. Biol. Chem. 244, 1081-1083.
13. Umbreit, W.W., Burris, R.H., and Stauffer, J.F. (1972) Manometric and Biochemical Techniques, 5th ed., 387pp, Burgess Publishing Co., Minneapolis, Minn.
14. Bowes, G., and Ogren, W.L. (1972) J. Biol. Chem. 247, 2171-2176.
15. Jensen, R.G., and Bassham, J.A. (1968) Proc. Nat. Acad. Sci. U.S., 56, 1095-1101.
16. Dixon, M., and Webb, E.C. (1964) The Enzymes, 2nd ed, pp. 84-87, Longmans, Green and Co., London.
17. Tolbert, N.E. (1971) Photosynthesis and Photorespiration, Hatch, M.D., Osmond, C.B., and Slatyer, R.O. Eds., pp. 458-471, Wiley - Interscience, New York.
18. Ludwig, L.J., and Canvin, D.T. (1971) Can. J. Bot. 49, 1299-1313.
19. D'Aoust, A.L., and Canvin, D.T. (1973) Can. J. Bot. 51, 457-464.
20. Wilkinson, G.N. (1961) Biochem. J. 80, 324-332.