CARBON DIOXIDE INCORPORATION BY CHLOROPLAST EXTRACTS AT HIGH pH

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Received 22 October 1973

1. Introduction

The recently discovered oxygenase action of the enzyme ribulose 1,5-diphosphate (RuDP) carboxylase [1, 2] has provided a rational explanation for the observed inhibitory effect of oxygen on CO_2 fixation by isolated chloroplasts [3] in terms of competition of O_2 and CO_2 for the substrate RuDP. As the oxygenase activity has a pH optimum around 9.3 [2], while that of the carboxylase is near to 8 [4], it would be expected that oxygen inhibition would become more marked at high pH. Experiments described here have shown that when ribose 5-phosphate (R-5P) and ATP are added as substrates to chloroplast extracts, the carboxylation reaction has an unexpectedly high pH optimum, and oxygen inhibition does not increase significantly in this system over the pH range 8–9. Further, at pH values around 9 the carboxylation reaction shows a K_M (CO_2) which is considerably lower than that at pH values nearer neutrality, and which approaches the value observed with intact leaves.

2. Experimental

Chloroplasts were isolated from young leaves of spinach plants grown in liquid culture in a glasshouse. After 10 min illumination at 4°C, 15 g leaf was blended for 4 sec into 40 ml extraction buffer (essentially Buffer A of Jensen and Bassham [5] with nitrate omitted), filtered through three layers of Miracloth, and centrifuged 8 sec at 2000 *g*. The pellet was resuspended in 10 ml of the same buffer and resedimented at 2000 *g* for 4 sec. Chloroplasts thus prepared were photosynthetically highly active, giving CO_2 fixation rates of 250 to 350 μmol CO_2/mg chlorophyl/hr when assayed either by incorporation of $^{14}CO_2$ or by CO_2 dependent O_2 evolution.

To prepare the chloroplast extract, the pellet (approx. 0.2 ml) was suspended for 10 min at 0°C in 1 ml 0.05 M Tris–SO_4 buffer, pH 7.6, centrifuged at 5000 *g* for 5 min, and the supernatant passed through a column of Sephadex G-25 equilibrated with the same Tris–SO_4 buffer. The emergent protein peak was collected (1.8–2.0 ml, A = 6–8 at 275 nm) and made 2 mM with dithiothreitol.

Assays of CO_2 fixation were carried out at 25°C under O_2 or N_2 in a mixture containing 0.1 ml H_2O, 0.1 ml buffer, 25 μl chloroplast-extract, 10 μl $NaH^{14}CO_3$, 10 μl ATP (250 mM) and 10 μl R-5P (100 mM). The buffer contained Tris (hydroxymethyl) methylaminopropane sulphonic acid (TAPS) 0.05 M, adjusted to the required pH with KOH, 20 mM $MgCl_2$ and 50 mM NaCl; the $NaH^{14}CO_3$ concentration varied with the experiment. Aliquots (25 μl) were taken 1, 3, 5 and 7 min after addition of R-5P, mixed with 25 μl 50% acetic acid, and counted after drying on filter paper. The rates of fixation, linear over the period studied, are expressed in terms of the chlorophyll equivalent to the chloroplast extract used. This gives a measure of comparison with the performance of intact chloroplasts.

3. Results and discussion

The rates of CO_2 fixation at a range of pH values are given in table 1, showing that the rate of carboxyl-

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Table 1
Rate of CO_2 fixation (μmol CO_2/mg chlorophyll/hr) by chloroplast extracts.

	pH	Gas phase	Rate O_2	Rate N_2	Ratio $\frac{\text{Rate in } N_2}{\text{Rate in } O_2}$
(a)	8.25		23	36	1.56
	8.50		34	54	1.58
	8.70		46	71	1.54
	8.90		55	89	1.62
	9.05		61	105	1.72
	9.30		44	82	1.85
(b)	8.90		–	168	–
	9.05		–	178	–
	9.30		–	191	–

$NaH^{14}CO_3$ concentration in reaction mixture: (a) 8 mM; (b) 40 mM.

Table 2
K_M values for carboxylation reaction at different pH values.

pH	Gas phase	$K_M(HCO_3^-)$	$K_M(CO_2)$ in equilibrium with HCO_3^-
8.4	N_2	9 mM	100 μM
8.8	N_2	11.6 mM	44 μM
9.0	N_2	13.3 mM	33 μM
9.3	N_2	14.7 mM	17 μM
9.0	CO_2-free air	14.3 mM	35.5 μM

ation in the system under study increased with increasing pH, falling off above pH 9 at the lower $NaHCO_3$ concentration. The degree of O_2 inhibition rose appreciably only at pH 9 and above.

The K_M (HCO_3^-) of CO_2 fixation by the system was measured at several pH values using buffers prepared under N_2 and stored in a CO_2-free atmosphere. Allowance was made for the K_2CO_3 contributed by the Analar KOH used. Lineweaver–Burk plots were used to derive the K_M values given in table 2, both for bicarbonate and for the actual substrate, dissolved CO_2 [6] in equilibrium with these bicarbonate concentrations at each pH [7].

The actual pH at which the maximum rate of CO_2 fixation occurs has not been established, but in the presence of high levels of bicarbonate it is certainly above 9. This high value, comparable to that of the oxygenase activity, explains the relative constancy of oxygen inhibition observed. At the point where the inhibition increased (pH 9.05) the 8 mM bicarbonate present was considerably below the K_M value (approx. 14 mM), severely limiting the carboxylation rate.

The question arises as to whether the high rates of in vitro carboxylation observed here can be related to those occurring in vivo; in other words, can the stromal pH in the chloroplast reach values above 9? It is widely considered that illumination will render the stromal region more alkaline [8] but the magnitude of the change is less documented. Stromal pH values from 6.8 to 8.8 have been reported for chloroplasts in the dark [9] and upon illumination these may become even higher, so it appears not to be impossible that light driven photosynthetic carboxylation may take place at pH values above 9.

The relatively low measured values of K_M (CO_2) approach the concentration in solution in equilibrium with atmospheric CO_2 (approx. 10 μM at 25°C), at which concentration both plants and isolated chloroplasts fix CO_2 at about half their maximum rate [10]. This CO_2 concentration is independent of pH, although the bicarbonate in equilibrium with it will increase greatly under alkaline conditions [11]. Previous in vitro studies of carboxylation by chloroplast derived systems have approached 10 μM for K_M (CO_2) only when freshly lysed chloroplasts were studied, and the K_M value rose on further disruption of the chloroplasts [12].

More commonly observed values of K_M (CO_2) for in vitro carboxylation stages of the Calvin cycle by chloroplast extracts or by purified fractions range from 0.18 mM (calculated from Walker [13]) to considerably higher values [6], and this has for some time presented an enigma as to how the recognized carboxylation mechanisms in vitro could account for the observed performance in vivo. Most results in the literature, however, have been recorded for in vitro systems around pH 8 or below; so the observations reported here at high pH values may resolve the discrepancy. Provided that it can be substantiated that the stromal pH can exceed 9, the in vitro performance of an extract from chloroplasts appears consistent with that in vivo.

The increasing rates of carboxylation at higher pH values in this system were observed only when R-5P and ATP were supplied as substrates; when RuDP was substituted for R-5P + ATP the rate of carboxy-

lation was somewhat higher around pH 8, but fell off above pH 8.4 and was about one fifth of the R-5P + ATP value at pH 9. It is possible that this difference in performance of the two substrates is due to the existence of an activatable CO_2-fixing complex as postulated by Müller [14]. If so, it was extracted in a fully activated form, as the chloroplasts were obtained from leaves which were illuminated prior to maceration.

The physical demonstration of the postulated complex has not been attempted in this work; but since the activity of the chloroplast extracts was not modified by precipitation with ammonium sulphate (75% sat.) prior to passage through G25, any such complex would appear to be reasonably stable.

Acknowledgements

Grateful thanks are due to members of the Plant Industries Division, CSIRO, Canberra, for providing such excellent facilities and critical encouragement.

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