RIBULOSE BISPHOSPHATE CARBOXYLASE – LACK OF DARK INACTIVATION OF THE ENZYME IN EXPERIMENTS WITH PROTOPLASTS

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Received 25 October 1978

1. Introduction

Although early measurements of ribulose bisphosphate (RuBP) carboxylase (EC 4.1.1.39) suggested that there was insufficient activity to account for observed rates of photosynthesis, subsequent experiments have shown that the enzyme is inactivated in the absence of CO_2 and Mg^{2+} [1–4]. The enzyme can be reactivated by incubation with CO_2 and Mg^{2+} and the experiments in [5] suggest that the enzyme first reacts slowly with CO_2 (eq. (1)) to form an inactive enzyme–CO_2 complex which subsequently reacts rapidly with Mg^{2+} to form the active complex, enzyme–CO_2–Mg^{2+} (eq. (2)).

$$\text{Enzyme (inactive)} + CO_2 \rightleftharpoons \text{enzyme–}CO_2 \text{ (inactive)} \quad (1)$$

$$\text{Enzyme–}CO_2\text{ (inactive)} + Mg^{2+} \rightleftharpoons \text{enzyme–}CO_2\text{–}Mg^{2+}\text{ (active)} \quad (2)$$

$$\text{Enzyme–}CO_2\text{–}Mg^{2+} + CO_2 + \text{RuBP} \rightleftharpoons \text{enzyme–}CO_2\text{–}Mg^{2+} + 2\text{(3-phosphoglycerate)} \quad (3)$$

The actual rate of catalysis (eq. (3)) of the enzyme depends on its activation state and on the pH and the concentrations of CO_2 and RuBP. In addition, certain metabolites are thought to increase the activity of the enzyme [15].

From measurements of the levels of RuBP during light–dark transitions in algae and isolated chloroplasts, it has been suggested that RuBP carboxylase is light activated in vivo and that its activity is decreased in the dark [7,8]. When chloroplasts are illuminated, the pH and Mg^{2+} concentration increase in the stromal compartment and there are changes in the metabolite levels so any or all of these changes could bring about an increase in RuBP carboxylase activity [1,2]. It is still unclear, however, to what extent the enzyme is light activated in vivo and, of equal importance, whether it is inactivated in the dark. In isolated spinach chloroplasts it was found [9] that the enzyme was in a relatively active state but could be deactivated and activated whilst still in the chloroplast by altering the concentration of CO_2 in the medium. In addition, the activity of the enzyme was increased 1.2–1.5-fold when the chloroplasts were illuminated. The activity of RuBP carboxylase was found [10] to be increased 2.0–4.3-fold by illumination; orthophosphate was required for this light activation. The possibility exists, however, that the activation state of the enzyme may alter during chloroplast isolation.

To further clarify this situation, we have measured RuBP carboxylase in freshly-ruptured protoplasts. There was no significant increase in enzyme activity when the protoplasts were illuminated. Moreover the activity in protoplasts from the dark was more than sufficient to account for the observed rates of photosynthesis. This suggests that inactivation of RuBP carboxylase does not occur to any significant extent in vivo.

2. Materials and methods

Protoplasts of wheat (*Triticum aestivum*) or barley (*Hordeum vulgare*) were prepared and purified as in [11]. The protoplasts were stored on ice in a medium

containing 500 mM sorbitol, 1 mM $CaCl_2$, 5 mM MES (pH 6.0) and were normally used within 3–4 h. For the isolation of intact chloroplasts, protoplasts were sedimented at 100 × *g* for 3 min and resuspended in a medium containing 400 mM sorbitol, 4 mM EDTA, 25 mM Tricine (pH 8.0). The protoplasts were ruptured by 3 passes through a syringe with a 20 μm nylon mesh covering the end and the chloroplasts were separated by centrifugation at 500 × *g* for 1 min and resuspended in the above medium. The percentage of intact chloroplasts were measured by the ferricyanide method [12] and chlorophyll (chl.) was determined by the method in [13].

Photosynthesis was measured by adding protoplasts to a medium containing 500 mM sorbitol, 1 mM $CaCl_2$, 2.5 mM $NaHCO_3$, 25 mM Tricine, pH 7.6 or pH 8.0, and determining the rate of oxygen evolution as in [11]. Oxygen evolution by chloroplasts was measured in a medium containing 400 mM sorbitol, 4 mM EDTA, 0.3 mM P_i, 2.5 mM $NaHCO_3$, 25 mM Tricine (pH 8.0). Chlorophyll was 40–50 μg/ml and the measurements were made at 20°C.

To measure RuBP carboxylase activity a sample of the suspension of protoplasts or chloroplasts, preincubated as for oxygen measurements, was rapidly mixed into a hypotonic medium containing detergent, RuBP and $^{14}CO_2$. Normally, 50 μl (~2.5 μg chl.) of the suspension of protoplasts or chloroplasts was added to 450 μl of hypotonic medium to give the following final concentrations: 0.1 M Tris–HCl (pH 8.1); 5 mM $MgCl_2$, 0.5 mM RuBP, 0.25% (v/v) Triton X-100 and 1.26 mM $NaH^{14}CO_3$. Samples (50 μl) were withdrawn every 15 s and immediately mixed with 500 μl 10% propionic acid and then dried on planchettes. Total radioactivity was determined at the end of each experiment by adding a 50 μl sample to 950 μl 50 mM Tris base and 20 μl of this suspension was added to 200 μl of 0.1 M NaOH and dried on a planchette. Radioactivity was determined with a Nuclear Chicago gas flow counter. The concentration of bicarbonate in the assay medium for RuBP carboxylase was corrected for CO_2 transferred with the protoplast or chloroplast suspension. If the protoplasts or chloroplasts were illuminated during the preincubation, the concentration of $NaHCO_3$ was increased slightly so that the same amount of CO_2 was transferred whether the samples were preincubated in darkness or in the light.

3. Results

When protoplasts were rapidly mixed into a medium containing detergent, RuBP and $^{14}CO_2$, the incorporation of CO_2 was linear for at least 60 s (fig.1) indicating that a rapid lysis of the protoplasts had occurred. A lag of 1–3 s was observed reflecting the time taken for mixing and complete rupture of the protoplasts. No inhibitory effect of Triton X-100 was observed at $\leqslant 0.5\%$ but non-linear CO_2 fixation occurred with $< 0.2\%$ indicating a relatively slow lysis of the protoplasts. The linearity of the CO_2 fixation shows that the activation state of the carboxylase did not change to any great extent during the assay. In the absence of RuBP, CO_2 fixation was $< 1\%$ of the standard assay demonstrating that endogenous substrates did not contribute significantly to the reaction. The standard assay contained suboptimal levels of CO_2 (1.26 mM $NaHCO_3$ (pH 8.1)) and air levels of oxygen. If $NaHCO_3$ was increased to 5 mM, the RuBP carboxylase activity was approximately doubled to give values of 280–340 μmol. mg chl.$^{-1}$.h^{-1} for protoplasts which had been preincubated in the light. The maximum rates of photosynthesis by the protoplasts were 120–180 μmol. mg chl.$^{-1}$.h^{-1} in saturating CO_2.

When protoplasts were ruptured and then incubated for 6 min in the absence of added CO_2 and Mg^{2+}, the RuBP carboxylase activity was decreased to 2–3% of that in freshly-lysed protoplasts demonstrating that the enzyme could be readily inactivated in vitro. However, when intact protoplasts were incubated in the absence of added CO_2, 75–85% of the activity remained after 6 min and this decrease was prevented by including CO_2 in the medium. Similar results have been obtained [9] with RuBP carboxylase in isolated chloroplasts. Illumination of the protoplasts only slightly increased the activity of RuBP carboxylase. Table 1 gives rates of oxygen evolution and RuBP carboxylase activities for different preparations of wheat protoplasts. In each case, the activity of RuBP carboxylase in dark pretreated protoplasts (measured at pH 8.1, 1.26 mM $NaHCO_3$) was greater than the maximum rate of oxygen evolution (measured at pH 7.6, 2.5 mM $NaHCO_3$) and the enzyme activity was not greatly increased by illumination of the protoplasts. The wheat was normally illuminated

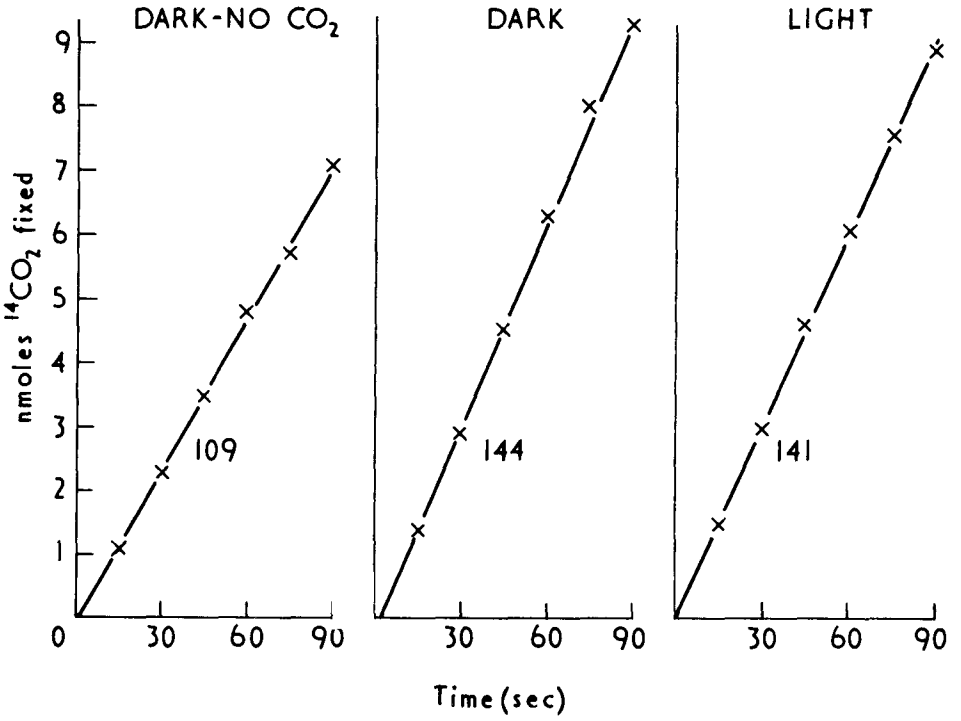

Fig.1. Fixation of CO_2 by protoplasts following lysis in a hypotonic medium containing detergent, RuBP and $^{14}CO_2$. Protoplasts were isolated from wheat which had been kept in darkness for 24 h. The protoplasts were preincubated for 6 min at pH 7.6 in the presence or absence of 2.5 mM $NaHCO_3$ in the dark or light. The rate of CO_2-dependent oxygen evolution by the protoplasts under the same conditions was 115 μmol. mg chl.$^{-1}$.h^{-1}. The rates of $^{14}CO_2$ fixation are given for each experiment, expressed as μmol. mg chl.$^{-1}$.h^{-1}.

Table 1
Rates of CO_2-dependent oxygen evolution and the effect of illumination on RuBP carboxylase activity of wheat protoplasts

Exp.	Oxygen evolution	RuBP carboxylase		Ratio light/dark
		Dark	Light	
1	139	182	228	1.25
2	152	161	186	1.16
3	167	174	206	1.18
4	136	150	207	1.38
5	117	165	166	1.01
6	115	144	141	0.98

RuBP carboxylase was measured in protoplasts after 6 min preincubation in the dark or light under the same conditions as for oxygen evolution (2.5 mM $NaHCO_3$, pH 7.6). In exp. 5 and 6, the wheat was kept in darkness for 24 h prior to isolating the protoplasts. Rates are expressed in μmol. mg chl.$^{-1}$.h^{-1}

during digestion [11] which may have fully activated the carboxylase. Accordingly, in an attempt to inactivate the enzyme, protoplasts were isolated in the dark from leaves which had been kept in darkness for 24 h prior to the experiment. If anything, there was less light activation of RuBP carboxylase in protoplasts from dark pretreated tissue than in normal preparations (table 1, fig.1).

Table 2 shows the results of an experiment where the concentration of $NaHCO_3$ was reduced so that photosynthesis by the protoplasts became CO_2-limited and the concentration of $NaHCO_3$ in the carboxylase assay was reduced to 0.77 mM. There was a decrease in carboxylase activity but the ratio of activity between light and dark preincubated protoplasts was not significantly affected. Thus even when CO_2 is limiting, the enzyme activity was not greatly increased by illumination of the protoplasts. Chloro-

Table 2
Rates of CO_2-dependent oxygen evolution and activity of RuBP carboxylase in wheat protoplasts and chloroplasts

Material	Preincubation	Oxygen evolution	RuBP carboxylase		Ratio light/dark
			mM $NaHCO_3$	Rate	
Protoplasts	None	–	1.26	173	–
Protoplasts	Dark, 2.5 mM $NaHCO_3$	–	1.26	150	–
Protoplasts	Light, 2.5 mM $NaHCO_3$	136	1.26	207	1.38
Protoplasts	Dark, 1 mM $NaHCO_3$	–	0.77	93	–
Protoplasts	Light, 1 mM $NaHCO_3$	73	0.77	136	1.46
Chloroplasts	None	–	1.26	144	–
Chloroplasts	Dark, 2.5 mM $NaHCO_3$	–	1.26	138	–
Chloroplasts	Light, 2.5 mM $NaHCO_3$	120	1.26	172	1.25

The chloroplasts were isolated from the protoplasts as in section 2 and were 78% intact. Samples for the carboxylase assay were taken after 10 s incubation or following a 6 min preincubation in dark or light at pH 8.0 with 2.5 mM or 1.0 mM $NaHCO_3$. RuBP carboxylase activity was measured at pH 8.1. Rates are expressed as μmol. mg chl.$^{-1}$.h^{-1}

plasts isolated from the protoplasts contained lower activities of RuBP carboxylase on a chlorophyll basis (table 2) but if the values were corrected for the percentage of broken chloroplasts, which would have lost most, or all, of their carboxylase activity [3,12] almost complete recovery of carboxylase activity in the chloroplast preparation was observed. The increase in carboxylase activity following illumination of the chloroplasts was similar to that observed with the protoplasts (table 2).

In attempts to activate the enzyme fully, protoplasts were ruptured and then incubated for 6 min at pH 8.1 with 5 mM $NaHCO_3$ and 20 mM $MgCl_2$. From such experiments, it was estimated that 80–100% of the carboxylase from protoplasts preincubated in the light was in a fully active state. It seems likely that the enzyme exists in vivo in the dark as enzyme–CO_2 which is only converted to the active form, enzyme–CO_2–Mg^{2+} by light-dependent increases in the stromal Mg^{2+} concentration. Because the standard assay contained $MgCl_2$, any enzyme–CO_2 released from the protoplasts would be rapidly converted to the active complex and the assay would measure enzyme–CO_2 plus enzyme–CO_2–Mg^{2+}. When $MgCl_2$ was omitted from the assay, CO_2 fixation was only linear for 30–45 s before a significant decrease in rate was observed which probably reflected the dissociation of the active complex in the absence of Mg^{2+}. Nevertheless, the initial rate was only slightly decreased in comparison to the normal assay and it was estimated that only 12–15% of the activity in the standard assay resulted from activation of enzyme–CO_2. Thus it appears that much of the enzyme is in a fully active state in protoplasts, even in the dark and that there is no major increase in the activation state of the enzyme upon illumination.

4. Discussion

In any consideration of RuBP carboxylase, it is important to distinguish between factors which affect the activation state of the enzyme and those which alter the actual rate of catalysis. The situation is further complicated by the fact that some factors (e.g., CO_2 concentration and pH) can affect both processes. When a plant is transferred from darkness to light, it is most unlikely that the concentration of CO_2 and bicarbonate in the chloroplasts will increase. Moreover, the initial light-dependent alkalisation of the stroma will tend to cause a transient decrease in the concentration of free CO_2, the species which is responsible for both activation and catalysis [5]. Thus any increase in the activation state of the carboxylase immediately following illumination is more likely to result from increases in stromal pH and Mg^{2+} concentration than from CO_2. Our own results suggest that RuBP carboxylase is not inactivated to any great

extent in vivo and that, as this would imply, there is no correspondingly large activation upon illumination. This would be readily understandable if the Mg^{2+} concentration within the stroma remained sufficiently high, even in the dark, to prevent dissociation of the active complex, enzyme–CO_2–Mg^{2+}.

Although these measurements relate to the activation state of the enzyme, it must be stressed that the actual rates of catalysis will also be governed by the pH and the concentrations of CO_2 and RuBP in the stroma. Whilst the assay conditions were chosen to approximate to those thought to obtain in the illuminated stroma, the actual values (particularly the concentration of CO_2) at the site of catalysis could be quite different. Thus the rate of catalysis of the enzyme could be increased in the light as a result of light-induced increases in stromal pH. As for some other enzymes of the reductive pentose phosphate pathway, there may then be no question that catalysis is more effective in the environment provided by the illuminated stroma. What is less certain is the degree to which activation, as distinct from a change towards optimal conditions for catalysis, is important in this regard. Even more doubtful is the contention that the organism will necessarily derive some advantage from dark de-activation of the enzyme concerned.

The maximum rate of photosynthesis by whole leaves, isolated chloroplasts [1] or protoplasts [11] is not achieved until some minutes after the illumination is started. This induction phase has been attributed to an autocatalytic build-up of photosynthetic intermediates, possibly modulated by the activation of some catalyst [14,15]. From the present measurements it is clear that there is little change in the activation of RuBP carboxylase during this period in which photosynthesis rises from zero to its maximum value.

As in [10] we have observed some increases in carboxylase activity during illumination of spinach chloroplasts but the ease with which artifactual de-activation can be brought about in these systems dictates an additional degree of caution in any attribution of physiological significance. This is particularly true of induction where an initial lag in photosynthetic carbon assimilation may be ascribed, in part, to light activation of catalysis. It seems unlikely, however, that activation of the carboxylase can play any part in induction in wheat and barley or that it can play a major role in spinach where its activity during the first few minutes of illumination is substantially greater than that needed to support the observed rates of photosynthesis.

We do not dispute that, under certain conditions, some light activation of the carboxylase can be observed in vitro. We are, however, increasingly inclined to the view that this is normally limited in its extent and may do little more than repair unintentional inactivation, during isolation, of a sort which is so dramatically accomplished by deliberate short term incubation in media containing no added CO_2 or Mg^{2+}.

Acknowledgements

Financial support was provided by the UK Science Research Council and The Rank Prize Fund. We are grateful to Jackie Rowell for expert technical assistance.

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