Ribulose biphosphate carboxylase/oxygenase in toluene-permeabilized *Rhodospirillum rubrum*

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Toluene-permeabilized *Rhodospirillum rubrum* cells were used to study activation of and catalysis by the dual-function enzyme ribulose biphosphate carboxylase/oxygenase. Incubation with CO₂ provided as HCO₃[–], followed by rapid removal of CO₂ at 2°C and subsequent incubation at 30°C before assay, enabled a determination of decay rates of the carboxylase and the oxygenase. Half-times at 30°C with 20 mM-Mg²⁺ were 10.8 and 3.7 min respectively. Additionally, the concentrations of CO₂ required for half-maximal activation were 56 and 72 μM for the oxygenase and the carboxylase respectively. After activation and CO₂ removal, inactivation of ribulose biphosphate oxygenase in the presence of 1 mM- or 20 mM-Mn²⁺ was slower than that with the same concentrations of Co²⁺ or Mg²⁺. Only the addition of Mg²⁺ supported ribulose biphosphate carboxylase activity, as Mn²⁺, Co²⁺ and Ni²⁺ had no effect. A pH increase after activation in the range 6.8–8.0 decreased the stability of the carboxylase but in the range 7.2–8.0 increased the stability of the oxygenase. With regard to catalysis, *K_m* values for ribulose 1,5-bisphosphate^{4–} were 1.5 and 67 μM for the oxygenase and the carboxylase respectively, and 125 μM for O₂. Over a broad range of CO₂ concentrations in the activation mixture, the pH optima were 7.8 and 8–9.2 for the carboxylase and the oxygenase respectively. The ratio of specific activities was constant (9:1 for carboxylase/oxygenase) of ribulose biphosphate carboxylase/oxygenase in toluene-treated *Rsp. rubrum*. Below concentrations of 10 μM-CO₂ in the activation mixture, this ratio increased.

Ribulose biphosphate (RuBP) carboxylase (EC 4.1.1.39) catalyses primary CO₂ assimilation in autotrophic bacterial species (McFadden, 1973). In 1971 a second catalytic activity in this protein was implied (Ogren & Bowes, 1971) and later was established to be a mono-oxygenase that catalysed the oxygenolysis of RuBP to phosphoglycerate and phosphoglycollate (Andrews *et al.*, 1973; Lorimer *et al.*, 1973). In recent years it has become evident that activation of plant RuBP carboxylase is initiated by slow binding of CO₂ and completed by rapid binding of Mg²⁺ (Lorimer *et al.*, 1976). Activation of the oxygenase appears to be similar (Badger & Lorimer, 1976). Although less is known about activation of the bacterial enzyme, both carboxylase and oxygenase activities are detectable.

Abbreviation used: RuBP, ribulose 1,5-bisphosphate.

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Moreover, a variety of quaternary structures is found from bacteria, ranging from those containing only large (L) 55 000-dalton subunits to those also containing small (S) 15 000-dalton subunits in L₈S₈ combinations like the plant enzymes (McFadden, 1980). In particular, the enzyme from *Rhodospirillum rubrum*, one of the non-sulphur photosynthetic bacteria, is the simplest known and is a dimer lacking small subunits (Tabita & McFadden, 1974b). Thus the Prokaryota afford a diversity of RuBP carboxylase/oxygenases enabling varied comparisons of the relationship between structure and function and for both activities.

Compelling evidence suggests that RuBP oxygenase initiates photorespiration by oxidative production of phosphoglycollate, the substrate of photorespiration (Andrews & Lorimer, 1978). Thus an enigma exists in that a single protein, RuBP carboxylase/oxygenase, may contribute to both photosynthesis and photorespiration, which are

fundamentally opposed processes. This mode of function is unprecedented in biology and raises intriguing questions about the prospects and consequences of controlling RuBP carboxylase/oxygenase ratios in plants (McFadden, 1980).

In this broad context we have chosen to study *Rsp. rubrum* as a model for higher plants. Indeed, the excretion of glycolate by this organism is light- and O_2 -dependent, and is inhibited by CO_2 . Moreover, the excretion of glycolate correlates with the RuBP carboxylase/oxygenase content of toluene-permeabilized cells (Storrø & McFadden, 1981). In the present studies toluene-treated cells were used in anticipation that the active form of the enzyme ($E \cdot CO_2 \cdot Mg^{2+}$) would have a longer lifetime after removal of activator CO_2 . We now describe measured decay rates of both RuBP carboxylase and RuBP oxygenase and additional characterization of this dual-function enzyme in toluene-permeabilized cells.

Materials and methods

Chemicals

The following special reagents were commercial preparations: tetrasodium D-ribulose 1,5-bisphosphate and bovine serum albumin from Sigma Chemical Co.; Ascarite (a sodium hydrate asbestos absorbent of CO_2) from A. H. Thomas Co.; $NaH^{14}CO_3$ from ICN Chemical Radioisotope Division; and Bicine [*N,N*-bis-(2-hydroxyethyl)-glycine], Mes (4-morpholine-ethanesulphonic acid), Caps [3-(cyclohexylamino)propanesulphonic acid] and Mops (4-morpholinepropanesulphonic acid) from Calbiochem—Behringer Corp. All more common compounds were of reagent-grade quality. Purity of chemicals as indicated by the vendor was taken into account in calculating concentrations.

Buffers

Buffers were made CO_2 -free by adjusting the pH to 3–4 and boiling under reflux for 15 min. Rapidly cooled buffers were stored in stoppered bottles. Before use, the pH was adjusted with CO_2 -free NaOH.

Growth of *Rsp. rubrum*

Cells were grown in bicarbonate-limited medium and harvested as described previously (Storrø & McFadden, 1981). The cells were washed and suspended, usually in 100 mM-Bicine/NaOH buffer, pH 8.0, to a final concentration of approx. 20 mg dry wt./ml.

Toluene treatment and activation of RuBP carboxylase/oxygenase

One volume of cell suspension (2.5–10 ml) and 0.5 vol. of toluene were placed in a screw-cap tube, and the tube was inverted gently several times during

2 min and placed in ice for 10 min (Tabita *et al.*, 1978). The cell suspension, which had changed from red to grey-red, was withdrawn with the underlayer by using a Pasteur pipette and stored at 2°C. Permeabilization was considered complete if the cell suspension revealed no inhibition by light of O_2 consumption (Storrø & McFadden, 1981). The toluene-treated cell suspension was then activated at pH 8.0 and 30°C for 10 min with $NaHCO_3$ (100 mM) and $MgCl_2$ (20 mM) in 100 mM-Bicine buffer. The activated cells were centrifuged at 2°C in a Beckman Microfuge B for 65 s, the supernatant was shaken out completely and the packed cells were immediately washed and resuspended in ice-cold CO_2 -free 100 mM-Bicine buffer, pH 8.0, containing $MgCl_2$ (20 mM) (BM buffer) to a concentration of approx. 20 mg dry wt./ml. The pH was checked regularly in the supernatants to ensure accurate pH measurements during the activation procedure. We estimate that the extracellular concentration of HCO_3^- carried over through cell washing and into the final suspension (20 mg/ml) was a maximum of 0.4 μ M (equivalent to 8 nM- CO_2 ; Umbreit *et al.*, 1964).

Assay of RuBP carboxylase activity

Carboxylase activities were determined in two ways. The RuBP-dependent carboxylase activity was determined with toluene-treated cells that had been activated (with HCO_3^-/Mg^{2+}), washed with BM buffer and incubated at 30°C with RuBP (pre-wash activation assay). The other determination was done on the same batch of toluene-treated, activated and washed cells, but this time the cells were re-activated with $NaHCO_3$ (50 mM) at 30°C to ensure complete activation (post-wash re-activation assay). Control experiments, without added RuBP, were performed for both types of assays, and the activity found was subtracted as background.

In the pre-wash activation assay the toluene-treated and activated cells were incubated at 30°C in CO_2 -free BM buffer for periods of 1, 2, 3, 4 and 5 min (10 mg dry wt. of cells/ml). The incorporation of $^{14}CO_2$ (McFadden *et al.*, 1975) was initiated by simultaneously adding $NaH^{14}CO_3$ and RuBP and was conducted under the following conditions: activated cells, 4.4 mg dry wt./ml; Bicine, 44.4 mM, pH 8.0; $MgCl_2$, 10 mM; $NaH^{14}CO_3$, 44.4 mM, with a specific radioactivity of 0.2 mCi/mmol; RuBP, 1.1 mM; total volume 0.225 ml. The reaction was stopped after 30 s by adding 0.1 ml of 50% (w/v) trichloroacetic acid. Unincorporated CO_2 was evolved overnight and the radioactivity of the samples was counted.

The pre-wash activation assay is summarized in Scheme 1.

In the post-wash re-activation assay, the toluene-

treated cells that had been activated and washed as just described above were re-activated with bicarbonate (50mM) for 10min at 30°C before incorporation was initiated by the addition of RuBP. The composition of the medium during incorporation, the incorporation time and the method of termination were the same as in the 'pre-wash activation assay'. Thus a direct comparison of the two assay methods could be made.

Assay of RuBP oxygenase activity

Oxygenase activity was determined as RuBP-dependent O₂ uptake in toluene-treated pre-wash activated cells by using a Hansatech oxygen electrode at 30°C. Electrode drift and RuBP-independent O₂ uptake were measured for 1 min before the addition of RuBP and subtracted from the O₂ uptake measured after the injection of RuBP. The samples were incubated at 30°C for 1, 2, 3, 4 and 5 min before RuBP (5 µl) was added to a total volume of 0.5 ml. Concentrations during incubation and assay were, activated cells, 4.0 mg dry wt./ml; MgCl₂, 20 mM; CO₂-free Bicine, 100 mM, pH 8.0; RuBP, 0.5 mM. The oxygen electrode was calibrated, and the toluene-treated, activated and washed cells were diluted in CO₂-free BM buffer saturated with air at 30°C. CO₂ had been removed by passing the air through a column (2 cm × 10 cm) of Ascarite, a wash-bottle of 1 M-NaOH and finally a wash-bottle of distilled water. Usually cells from the same activation batch were used for the two carboxylase determinations and the oxygenase determination. Protein was estimated from the cell density measured spectrophotometrically at 620 nm, for each oxygenase determination performed.

Determination of protein

Protein in whole cells was determined as described by Drews (1965), with bovine serum albumin as standard.

K_m^{RuBP} of RuBP oxygenase

Three samples of cells were toluene-treated, activated and washed. The cells were incubated for 1, 2 or 3 min before addition of different amounts of RuBP.

K_m^{RuBP} of RuBP carboxylase

The pre-wash activation assay was used. Three samples of cells were toluene-treated, activated and washed. The cells were incubated for 1, 3 or 6 min at 30°C before addition of NaH¹⁴CO₃ and different amounts of RuBP.

K_m^{O₂} of RuBP oxygenase

A Gilson Oxygenograph was used in these assays. CO₂-free BM buffers were bubbled with either CO₂-free N₂ or CO₂-free O₂ for 1 h at 30°C. The

CO₂ was removed from the gases as described above. The O₂ content of these solutions was determined relative to air at 30°C. Different O₂ concentrations were obtained by mixing the two buffers and the activated cell suspension.

Response to HCO₃⁻ in activation

Cells were washed and recovered in CO₂-free BM buffer and toluene-treated as described. Incubation was then conducted at different concentrations of NaHCO₃ for 10 min at 30°C before the washing and assay of the carboxylase and the oxygenase activities. Aside from a 5 s period before initiation of the assay, the concentrations of Bicine and Mg²⁺ were constant (see BM buffer) throughout the toluene treatment, incubation with bicarbonate, washing and assay. Assays at 30°C were initiated with NaH¹⁴CO₃ and RuBP, 5 s after the addition of supplementary Bicine and Mg²⁺ to maintain the concentrations of these components.

Oxygenase assays were performed as described above.

pH profiles for RuBP carboxylase and oxygenase

Cells were toluene-treated and activated for 10 min at pH 8.0 and 30°C as described for the 'pre-wash activation assay'. After one wash in BM buffer and preincubation at 2°C, pH ranges 6–7.5 and 8.5–10 were achieved by suspending cells at 30°C in CO₂-free Mes/NaOH and CO₂-free Caps/NaOH buffers respectively. An intermediate pH range was provided by Bicine buffers. The concentration of all three buffers was 100 mM, and all contained 20 mM-MgCl₂. To maintain the selected pH and the concentrations of buffer and MgCl₂ (at 100 mM and 20 mM respectively), a mixture of the appropriate buffer containing Mg²⁺ was added 5 s before initiation of carboxylase assays with NaH¹⁴CO₃ and RuBP. For the oxygenase assays, with a Hansatech oxygen electrode, cells in appropriate buffer were diluted with the same buffer and RuBP added. The pH was estimated for carboxylase assays by measuring the pH in an identical system containing non-radioactive bicarbonate and was measured directly after the oxygenase assays.

Experiments with metal ions

Cells were harvested, washed, toluene-treated and activated for 10 min with 150 mM-HCO₃⁻ at 30°C in the presence of 150 mM-Mops buffer, pH 7.0, and bivalent metal ion (1 or 20 mM). After being washed rapidly at 2°C once in the Mops buffer containing bivalent metal ion, but lacking HCO₃⁻, suspended cells were stored on ice for 1–24 min. One portion was then adjusted to 30°C for 1, 2, 3, 4 or 5 min before a 30 s assay for carboxylase initiated by addition of RuBP plus H¹⁴CO₃⁻ to concentrations of

1 and 20 mM respectively. At 5 s before initiation, sufficient Mops buffer and bivalent metal ion were added to maintain the concentrations during assay at 150 mM and 1 or 20 mM respectively. To assay for the oxygenase, cells in BM buffer were diluted with the same buffer, and RuBP was added to a final concentration of 1 mM. All other components, concentrations and conditions were identical with those just described above for the carboxylase assay.

For an alternative carboxylase assay, another portion of cells was activated for 10 min at 30°C with 45 mM- $\text{H}^{14}\text{CO}_3^-$, and the reaction was initiated with RuBP at a final concentration of 1 mM. The final concentration of $\text{H}^{14}\text{CO}_3^-$ during assay was 40 mM, and Mops buffer and metal ion concentrations were maintained throughout at 150 mM and 1 or 20 mM respectively.

Results

Decay of RuBP carboxylase and RuBP oxygenase after activation

In early experiments the washing step was conducted at 22°C in the pre-wash activation assay (Scheme 1). Results shown in Fig. 1 establish that the time course of incorporation of $^{14}\text{CO}_2$ was linear for durations up to 40 s. The observed rate was 67% of that obtained in analogous studies with the post-wash re-activation assay. Presumably, the

lower rate of fixation was due to leakage of CO_2 and resulting inactivation during washing at 22°C. For this reason in all subsequent experiments a 65 s washing step was conducted at 2°C (Scheme 1). It is

Fig. 1. Time course of incorporation of $^{14}\text{CO}_2$ after activation of toluene-permeabilized cells (Scheme 1), washing at 22°C (cf. Scheme 1) and incubation for 1 min before assay as indicated in Scheme 1

For experimental details see the text. For the line of best fit (shown), data distribute with a correlation coefficient of 0.985 in a linear-regression analysis.

Scheme 1. Summary of procedures used in the pre-wash activation assay
For full experimental details see the text. Abbreviation: Me^{2+} , bivalent metal ion.

important to stress that there was no re-activation of RuBP carboxylase during the routine 30s assay of this enzyme in any of the experiments described in the present paper.

Data in Fig. 2 establish that RuBP carboxylase is much more stable than RuBP oxygenase, in spite of the fact that toluene-treated cells had been activated under identical conditions before removal of exogenous CO_2 (provided as HCO_3^-). The respective half-times for activity decay were 10.8 and 3.7 min at 30°C .

In these experiments, the concentrations of CO_2 carried over into the mixture incubated at 30°C before assay were at most 4 and 1.6 nM for carboxylase and oxygenase experiments respectively. If it is assumed that RuBP carboxylase/oxygenase comprised 5% of the cellular protein in toluene-treated cells that had been grown on butyrate (Tabita & McFadden, 1974a), the internal concentrations of this dimeric 112000-dalton enzyme (Tabita & McFadden, 1974b) were 55 and $22\mu\text{M}$ in carboxylase and oxygenase experiments respectively. Thus during the pre-assay incubation at 30°C the concentration ratio of enzyme subunits to external CO_2 that had been carried over from activation was at least 27500:1 in experiments on both RuBP carboxylase and oxygenase. The rates of leakage of activating CO_2 from cells during washing in the cold and subsequent incubation at 30°C were not measured in the present work, nor was the rate of endogenous CO_2 generation. However, the latter was small, and in the long term dominated inactivation by leakage because 10h after incubation at 30°C an increase in RuBP carboxylase was measured that would have been expected with the addition of $50\mu\text{M}\text{-CO}_2$.

K_m values of the RuBP carboxylase and oxygenase activities

The double-reciprocal plots in Fig. 3 establish that a single K_m value of $14.7 (\pm 0.2)\mu\text{M}\text{-RuBP}$ can be obtained for RuBP oxygenase in toluene-treated *Rsp. rubrum*. Results also confirm that the oxygenase is sufficiently labile that a family of curves is obtained with slopes and intercepts that increase with incubation time at 30°C after activation. In effect each increase reflects a smaller concentration of active enzyme. In contrast, the analogous plots for the carboxylase assay are roughly superimposable (not shown), because that enzymic activity

Fig. 3. Double-reciprocal velocity-[substrate] plots for RuBP oxygenase

Velocities were determined in toluene-treated cells containing activated enzyme after incubation in CO_2 -free buffer at 30°C for: ■, 1 min; ▲, 2 min; ●, 3 min. For experimental details see the text.

Fig. 2. Specific activity of activated RuBP carboxylase (○) and RuBP oxygenase (●) as a function of pre-assay incubation time (Scheme 1) at 30°C in CO_2 -free Bicine buffer, pH 8.0, containing MgCl_2 (20 mM)

For experimental details see the text. Activity was measured in toluene-treated cells. □, RuBP carboxylase measured with fully activated enzyme.

is much more stable (see Fig. 2). The K_m was $670\mu\text{M}$ -RuBP. This value varied between 500 and $700\mu\text{M}$ in different experiments, but data scatter led to uncertainty in the K_m . In any case, the RuBP carboxylase K_m value was consistently 30–50-fold higher than that of the RuBP oxygenase.

The K_m for O_2 , based on extrapolation of data for post-activation incubation of 1, 2 and 3 min to zero time, was 0.125 mM.

Dependence of activation on CO_2

Because it was possible to activate both RuBP carboxylase and oxygenase, remove the CO_2 (provided as HCO_3^-) and still measure both activities after incubation at 30°C (Fig. 2), it was feasible to measure an overall response to CO_2 . In experiments in which the concentration of CO_2 (as HCO_3^-) was varied during activation at 30°C before its removal, fairly high proportions of carboxylase (45%) and oxygenase (23%) were found in cells that had been incubated in the absence of added CO_2 in comparison with those activated with 4 mM- CO_2 . Fig. 4 shows that the decays of the carboxylase and the oxygenase are markedly different after incubation of toluene-treated cells in the absence of CO_2 . Under these conditions, activities measured at zero time presumably reflect carry-over of endogenous CO_2 . In these experiments the time course of $^{14}\text{CO}_2$ incorporation during 30 s assay was linear (not shown) even after toluene-treated cells had been incubated at 30°C in the absence of HCO_3^- , i.e. toluene-treated cells had not been activated. This provided further evidence that measurable enzyme activation did not occur during the 30 s assay period.

pH-activity profiles determined in the presence of Mg^{2+}

Fig. 5 shows pH-activity profiles after activation at pH 8.0 for both RuBP carboxylase and RuBP

oxygenase. The pH optimum for the former was 7.8. In contrast, the pH optimum was broad and extended from approx. 8 to 9.2 for the oxygenase.

Fig. 5. Specific activities of RuBP carboxylase and RuBP oxygenase as a function of pH of incubation and assay at 30°C

Cells were toluene-treated and RuBP carboxylase/oxygenase was activated at pH 8.0. Cells were then incubated at 30°C in CO_2 -free buffer at the pH noted and activities were assayed at the same pH. Specific activities were calculated after extrapolation to zero-time incubation at 30°C as indicated in Fig. 2. For experimental details see the text. Precipitation in the mixture containing Mg^{2+} was detected above pH 9.5. Symbols: $\circ$, RuBP carboxylase; $\bullet$, RuBP oxygenase.

Fig. 4. Time courses for RuBP carboxylase and oxygenase in toluene-treated cells after incubation for 10 min at 30°C in the presence of various concentrations of CO_2 (provided as bicarbonate)

After incubation, HCO_3^- was removed as described and cells were incubated at 30°C for the time shown before assay for (a) RuBP carboxylase or (b) RuBP oxygenase. For experimental details see the text.

Dependence on metal ions

The decay of RuBP oxygenase as a function of metal ion present during and after activation at pH 7.0 is shown in Fig. 6. These experiments were conducted at pH 7.0 to avoid precipitation and/or oxidation of Mn^{2+} and Co^{2+} ions. Because CO_2 was present during activation but was removed before the subsequent timed incubation, the rates of decay presumably are a reflection of the dissociation of CO_2 from the enzyme. Active RuBP oxygenase is most stable in toluene-treated cells incubated with 1 mM- or 20 mM- Mn^{2+} and is of equivalent stability in the presence of 20 mM- Mg^{2+} or - Co^{2+} (Fig. 6). The enzyme decays most rapidly at 1 mM- Mg^{2+} and considerably less rapidly at 1 mM- Co^{2+} . Of interest is the fact that all activities are equivalent when

Fig. 6. Effect of bivalent metal ions on catalysis by RuBP oxygenase

Specific activities were determined in toluene-treated cells containing activated RuBP carboxylase/oxygenase after incubation in CO_2 -free buffer containing (a) 1 mM- or (b) 20 mM-metal ion (Me^{2+}) at pH 7.0. Symbols: ▲, Mn^{2+} ; ●, Mg^{2+} ; ■, Co^{2+} . For experimental details see the text.

extrapolated to zero time at a given metal ion concentration.

A comparison is made between the influence of metal ion at pH 7.0 on the oxygenase and the carboxylase activities in toluene-treated *Rsp. rubrum* in Table 1. It was noted that some RuBP carboxylase activity was detected after incubation in the complete absence of added metal ion (control). This was probably due to the retention by this enzyme of endogenous Mg^{2+} that was not removed in the washing process after the incubation with HCO_3^- . That this is the correct interpretation is indicated by the observation that incubation in the presence of 1 mM- or 20 mM- Ni^{2+} or 20 mM- Mn^{2+} yields no detectable RuBP carboxylase activity (Table 1). Presumably these ions at the concentrations indicated compete for endogenous Mg^{2+} but do not support activity. Of considerable interest, then, are the observations that Co^{2+} and Mn^{2+} are inactive in supporting activation of RuBP carboxylase in toluene-treated cells but that in the activation of RuBP oxygenase these two cations are equivalent to Mg^{2+} . Ni^{2+} does not support the activation of RuBP carboxylase or oxygenase (Table 1).

Ratios of RuBP carboxylase/oxygenase activities

The ratio of RuBP carboxylase/oxygenase activities can be varied in the range 9.2–17.5:1 (in the

Fig. 7. Ratio between specific activities of RuBP carboxylase and RuBP oxygenase as a function of different concentrations of bicarbonate during activation of RuBP carboxylase/oxygenase at pH 8.0 in toluene-treated cells (Scheme 1)

Specific activities were calculated after extrapolation to zero-time incubation at 30°C as indicated in Fig. 2. For experimental details see the text.

presence of Mg^{2+}) depending on the concentration of bicarbonate added in the activation mixture (Fig. 7). There is confirmation of the fact (cf. Fig. 4) that measurable activities occur in toluene-treated cells incubated in the absence of added CO_2 /provided as HCO_3^- .

pH profile for activation in the presence of Mg^{2+}

Half-lives of RuBP carboxylase and oxygenase as a function of pH are shown for experiments (Fig. 8)

Fig. 8. Half-lives of activated RuBP carboxylase/oxygenase in CO_2 -free buffer containing MgCl_2 (20 mM) at different pH values after activation (in toluene-treated cells) at pH 8.0 in the normal way

In washing, the pH was adjusted as indicated and maintained during enzymic assay. For experimental details see the text. Precipitation (probably due to the presence of Mg^{2+}) was detected above pH 9.5. Symbols: ○, RuBP carboxylase; ●, RuBP oxygenase.

in which Mg^{2+} was used for activation. The trends in response of RuBP carboxylase and oxygenase are opposed below pH 7.5 in cells that were treated identically during activation. Clearly the oxygenase became more stable as the pH was raised to 7.5, whereas the carboxylase became less stable.

Discussion

The activation of RuBP carboxylase from spinach (Lorimer *et al.*, 1976), soya bean (Laing *et al.*, 1975) and *Rsp. rubrum* (Christeller & Laing, 1978) is described below:

where C is CO_2 , Me is Mg^{2+} and E is enzyme, and equilibration of EC with Me is relatively fast compared with the first step. Activation of the second catalytic activity, RuBP oxygenase, follows the same sequence for the spinach enzyme (Badger & Lorimer, 1976) and both activities appear at the same rate with the spinach enzyme (Badger & Lorimer, 1976) or decay at the same rate with soya-bean RuBP carboxylase/oxygenase (Laing *et al.*, 1975). Unfortunately, considerably less is known about the activation of RuBP oxygenase from *Rsp. rubrum*. Tabita and co-workers have established that Mg^{2+} and HCO_3^- are required for the activation of the *Rsp. rubrum* oxygenase (Robison *et al.*, 1979; Martin & Tabita, 1981). Although CO_2 derived from HCO_3^- is almost certainly the activating species, as is the case for the spinach enzyme (Lorimer *et al.*, 1976), this has not been established for RuBP carboxylase/oxygenase from *Rsp. rubrum*.

Half-lives of soya-bean RuBP carboxylase have been estimated indirectly by measuring the decrease in rates of CO_2 fixation observed at HCO_3^- (CO_2) concentrations, which limited activation but were probably saturating with respect to catalysis (Laing

Table 1. Specific activities of RuBP carboxylase and oxygenase with 1 mM- and 20 mM-metal ion after activation in toluene-permeabilized *Rsp. rubrum*, removal of CO_2 for incubation at 30°C before assay

For experimental details see the text. The values of specific activity were obtained by extrapolation of decay curves (like those in Fig. 6) to zero time for all metal ion (Me^{2+}) concentrations and both enzymes. All incubations were conducted at pH 7.0.

Metal ion	RuBP carboxylase (nmol of CO_2 /min per mg of protein)			RuBP oxygenase (nmol of O_2 /min per mg of protein)		
	No Me^{2+}	1 mM- Me^{2+}	20 mM- Me^{2+}	No Me^{2+}	1 mM- Me^{2+}	20 mM- Me^{2+}
Mg^{2+}	—	55	140	—	6.3	12.9
Mn^{2+}	—	7.0	0	—	6.3	12.9
Co^{2+}	—	7.0	6.0	—	6.3	12.9
Ni^{2+}	—	0	0	—	0	0
Control	7.0	—	—	0	—	—

et al., 1975). In a single comparison, both RuBP carboxylase and oxygenase had identical half-lives (7 min) at 30°C at pH 8.5 in the presence of a single bicarbonate concentration of 1.0 mM. In the present experiments with toluene-treated *Rsp. rubrum*, direct estimates of half-lives were possible. For example, RuBP carboxylase/oxygenase could be activated, the activating HCO_3^- rapidly removed in the cold, and surviving activity measured in 30 s assays after prior incubation for various times at 30°C. The brief assay period chosen allowed no measurable reactivation of RuBP carboxylase during assay. Moreover, the effect of complete removal of HCO_3^- after activation could be examined and RuBP oxygenase could be assayed in the complete absence of exogenous inhibitory HCO_3^- .

The results obtained with toluene-permeabilized *Rsp. rubrum* are in striking contrast with limited data available for the higher-plant enzyme. By extrapolation to zero-time incubation at 30°C, it was determined that RuBP carboxylase and oxygenase have half-lives of 10.8 and 3.7 min respectively at 30°C at pH 8.0 in the presence of 20 mM- Mg^{2+} when incubated and assayed in the presence of 250 μM - O_2 . Of significance is the almost 3-fold difference observed in stability of these two activities. Less-direct evidence supporting greater instability of RuBP oxygenase was noted in studies of K_m values for RuBP in which slopes and y-intercepts in double-reciprocal plots increased as a function of pre-assay incubation time (Fig. 3).

A simple model that accounts for the observed differential decay of carboxylase and oxygenase activities is one in which there are two topographically distinct CO_2 -binding sites on each monomer, one site associated with activation of catalysis by RuBP carboxylase and the other with activation of catalysis by RuBP oxygenase. Whether this model is cogent only for the *Rsp. rubrum* dimer or for large subunits of the higher-plant-type enzymes as well remains to be seen.

The K_m values obtained for RuBP and O_2 in the presence of Mg^{2+} merit comment. In particular, the latter was obtained in the absence of added inhibitory CO_2 and was 125 μM (10% O_2), which is approx. 40% as high as that obtained by Christeller (1981). The apparent K_m values for RuBP were markedly different, and were 14.7 μM and 670 μM for RuBP oxygenase and carboxylase respectively. An analogous relationship was noted for both activities in the *Rsp. rubrum* and spinach enzymes (Martin & Tabita, 1981). When these values, which were measured in the presence of 10 mM- or 20 mM- Mg^{2+} , are corrected to K_m values for free RuBP^{4-} (Christeller, 1981), they are 1.5 μM and 67 μM for the oxygenase and the carboxylase respectively. The latter is about 8-fold higher than the analogous value measured in two different laboratories (Martin &

Tabita, 1981; Christeller, 1981). K_m for free RuBP^{4-} in the oxygenase reaction is in good agreement with the value reported for Mg^{2+} - RuBP^{4-} (Martin & Tabita, 1981) when that value is corrected to the concentration of free RuBP^{4-} . To recapitulate, K_m values for O_2 and RuBP^{4-} are in reasonable agreement when comparisons are made between toluene-treated cells and isolated RuBP oxygenase. In contrast, toluene-treated *Rsp. rubrum* cells yield a considerably elevated K_m for RuBP^{4-} in comparison with that for isolated RuBP carboxylase.

In spite of the differences noted in kinetic response to RuBP^{4-} and half-times of decay after activation, the ratio of velocities catalysed by RuBP carboxylase and oxygenase in toluene-treated cells is constant when 5–200 mM- HCO_3^- is used for activation (Fig. 7). Variations in cellular content of these two activities correlate closely with light- and O_2 -dependent glycolate excretion by *Rsp. rubrum* (Storrø & McFadden, 1981). This correlation and inhibition by CO_2 of glycolate excretion strongly suggest that the glycolate arises via glycolate phosphate from RuBP as a result of catalysis by RuBP carboxylase/oxygenase. Analogies to photorespiration have been discussed elsewhere (Storrø & McFadden, 1981). It is important to note, however, that the ratio of RuBP carboxylase/oxygenase activities in toluene-treated *Rsp. rubrum* increases when the concentration of HCO_3^- (CO_2) is decreased in the activation mixture (Fig. 7), suggesting further fundamental differences between the activation of these two catalytic activities.

Our studies of RuBP oxygenase in toluene-treated *Rsp. rubrum* were extended to include the effects of metal ion on the stability of this activity after activation and removal of HCO_3^- (CO_2). It was observed that the oxygenase decreases in stability in going from Mn^{2+} to Co^{2+} to Mg^{2+} (each at 1 mM). At 20 mM-metal ion, Mg^{2+} and Co^{2+} afford similar and increased stability, but Mn^{2+} considerably stabilizes RuBP oxygenase. Under these latter conditions the maximal velocities for all three cations are identical for RuBP oxygenase. This is in basic agreement with results obtained in other laboratories (Robison *et al.*, 1979; Christeller, 1981). Our failure to find activity with Ni^{2+} in assays of either the oxygenase or the carboxylase or with Co^{2+} in assays of the latter is in agreement with other investigations of the enzyme from *Rsp. rubrum* (Robison *et al.*, 1979; Christeller, 1981). In contrast, the present failure to find Mn^{2+} -supported activity of RuBP carboxylase in toluene-treated cells is markedly different from previously reported results for the enzyme isolated from either this organism (Robison *et al.*, 1979; Christeller, 1981) or higher plants (McFadden, 1980).

The pH profile for catalysis reveals a fairly sharp optimum for RuBP carboxylase of 7.8 and a broad

optimum at 8–9.2 for RuBP oxygenase in toluene-treated cells. These results are similar to those obtained with the enzyme isolated from *Rsp. rubrum*, although the pH optimum observed for the carboxylase was 7 (Kobayashi *et al.*, 1979). Of special interest, however, was the influence of pH on the stability of each activity in toluene-treated cells. In the pH range 6.8–8.0 the oxygenase doubled in stability, whereas between pH 7.2 and 8 the stability of the carboxylase decreased by approx. 25%.

In summary, our present results establish the utility of toluene-permeabilization of bacterial cells in studying RuBP carboxylase/oxygenase. They also imply that analogous investigations of toluene-treated chloroplasts may be of interest. Whether activation and catalysis of RuBP carboxylase/oxygenase in permeabilized cells (or organelles) are more reflective of those phenomena *in vivo* remains to be seen. Certainly, however, the present results establish that the activation and stability of these two activities are quite different in the enzyme from *Rsp. rubrum*. This further implies that their ratio may vary during growth and that it may be possible to alter the ratio by genetic manipulation.

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