Temperature Dependence of the Enzymic Carboxylation and Oxygenation of Ribulose 1,5-Bisphosphate in Relation to Effects of Temperature on Photosynthesis

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ABSTRACT

Carboxylase and oxygenase activities of ribulose bisphosphate carboxylase purified from wheat were measured over the temperature range 5 to 35°C either at constant O₂ and CO₂ concentrations or where the O₂ and CO₂ simulated the concentrations in water equilibrated at each temperature with the same gaseous phase. At constant CO₂ (14 micromolar) and O₂ (0.34 millimolar), the oxygenase to carboxylase ratio remained constant at 0.21 between 5 and 25°C but increased to 0.26 at 35°C. At O₂ and CO₂ concentrations near those expected in water equilibrated with air (21%/ v/v) O₂ containing 300 μl/l CO₂ at various temperatures, the ratio of oxygenase to carboxylase activity increased 2.2-fold between 15 and 35°C. At CO₂ and O₂ concentrations expected in water in equilibrium with substomatal concentrations of CO₂ in air (21%/ v/v) O₂, 210 μl/l CO₂, the oxygenase to carboxylase ratio increased from 0.25 at 10°C to 0.56 at 35°C. Between 20 and 30°C, the apparent Q₁₀ value for the oxygenase reaction was 1.78 and that for the carboxylase was 1.26. Hence, the different responses of photosynthesis and photorespiration to temperature are due more to changes in the relative solubilities of CO₂ and O₂ (the solubility ratio) than to changes in kinetic parameters of the reactions catalyzed by ribulose bisphosphate carboxylase.

In C₃ plants, O₂ inhibition of photosynthesis, the CO₂ compensation point, glycolate synthesis, and photorespiration are all increased with increasing temperature, more rapidly than net photosynthesis (12, 13, 15, 26) and a corresponding decrease in quantum efficiency is found (8). Attempts have been made to explain these observations in terms of the properties of RuBP-carboxylase/oxygenase (EC 4.1.1.39). Thus, if it is the oxygenase activity of RuBP carboxylase that results in synthesis of most of the substrate for photorespiration, namely phosphoglycolate (19), an increase in the oxygenase to carboxylase velocity ratio (vₒ/vₑ) in vivo with increased temperature could explain the observed effects on photorespiration and photosynthesis.

The velocity ratio vₒ/vₑ was reported to increase with temperature (2, 17) and the change was considered sufficient to account for increased photorespiration relative to photosynthesis.

Studies of the enzyme isolated from C₃ plants showed that the kinetic parameters of the oxygenase and carboxylase activities change differently as the temperature increases and this may explain changes in vₒ/vₑ and hence increased photorespiration (3, 17). Another proposal is that the increased solubility ratio O₂/CO₂ with increasing temperature may be a major cause of the increased photorespiration (16). Tenhunen et al. (24) have expressed doubts on this conclusion and have urged consideration of an empirical model. Badger and Collatz (3) proposed the use of partial pressures of CO₂ and O₂ in the gas phase as more appropriate measures than concentration in solution of the activities of CO₂ and O₂ in solution. However, treatments of enzyme kinetics conventionally use concentrations in solution and it is not helpful to introduce the alternative of partial pressures unless in a thorough treatment in terms of activities involving assessment of fugacities for the gaseous reactants. Calculations based on the data of Badger and Collatz (3) in terms of concentrations of CO₂ and O₂ in solution, compared to use of partial pressures of CO₂ and O₂ in equilibrium, confirm the significance of solubility ratio as the main cause of increase of photorespiration to photosynthesis with temperature. Reported kinetic parameters of RuBP carboxylase/oxygenase and the intercellular partial pressures of CO₂ and O₂ have been incorporated into mathematical models of photosynthesis and photorespiration, which also take into account limitations by the light reactions (9). It is important to determine whether the reaction kinetics based on concentrations of substrates in solution or gas solubility ratio is the most significant feature determining the effect of temperature on photosynthesis and photorespiration in order to make the models more accurate.

This paper describes the direct measurement in the same reaction mixtures of carboxylase and oxygenase activities under various defined conditions of temperature CO₂ and O₂ concentrations, using fully activated enzyme. This approach was used since measurement of the kinetic constants is complicated by the fact that the oxygenase reaction is not saturated at 100% O₂ at atmospheric pressure (1). The objective was to determine how vₒ/vₑ changes with temperature at constant concentrations of CO₂ and O₂, and hence where only changes in conventional parameters of enzyme kinetics are involved, as well as under conditions designed to simulate those expected in the chloroplast stroma where relative concentrations of gaseous substrates are also changed with temperature because of changes in solubility.

MATERIALS AND METHODS

RuBP Carboxylase from Wheat Leaves. The purified enzyme was available as a freeze-dried powder (5) and was activated by incubation for 1 h at 35°C in 100 mM Bicine-NaOH (pH 8.2) containing 10 mM NaHCO₃ and 20 mM MgCl₂. Subsequently, the enzyme solution was maintained at 25°C for the duration of the experiment. The specific activity of the preparations used was 0.5 to 0.9 μmol/min·mg protein at 25°C and 5 mM bicarbonate.

Preparation of Buffers. Bicine and MgCl₂ were added to distilled H₂O that had been boiled and then cooled under N₂. N₂ was bubbled through the solution for approximately 1 h before adjusting to the final pH with ‘CO₂-free’ NaOH (BDH). The solution

1 Abbreviation: RuBP, ribulose 1,5-bisphosphate.
was left with N₂ bubbling through at room temperature overnight before use. The same pH (8.20) was obtained at each temperature by mixing appropriate volumes of buffers of pH 8.20 at 5°C and 8.20 at 35°C (i.e. adjusted to 7.84 at 25°C and 8.38 at 25°C, respectively). The relative volumes of the two buffers needed was calculated on the basis that the pH for Bicine decreased by 0.09 for each 5°C rise in temperature. The calculation was checked by experiment and gave buffers at pH 8.2 at the various temperatures.

**Combined Assay for RuBP Carboxylase and Oxygenase.** Reaction mixtures were prepared in the oxygen electrode vessel (Hansatech Limited, King’s Lynn, Norfolk, U.K.) in a final volume of 0.5 ml. Assays were conducted at constant CO₂ (14 μM) and constant O₂ (0.34 mM) or with CO₂ and O₂ as calculated for water at the operating temperature equilibrated with natural air or subatmospheric levels of CO₂ in air (see Fig. 2).

(a) Assays at Constant CO₂ (14 μM) and Constant O₂ (340 μM). The concentration of O₂ was established by equilibrating the buffer at 35°C with 32.1% (v/v) O₂ (balance N₂). Aliquot of equilibrated buffer were transferred to the O₂ electrode and 5 min were allowed for the temperature to equilibrate with that of the surrounding water jacket. NaH¹⁴CO₃ was added to give 14 μM CO₂ in the final solution; the amount required was calculated from the dissociation constant appropriate to the chosen temperature and corresponding to an ionic strength of 0.1 (11). Allowance was made for CO₂ and HCO₃⁻ to be added with the activated enzyme.

(b) Assays at Air Concentrations of CO₂ and O₂. Buffer at the chosen temperature was equilibrated with CO₂-free air. Aliquots were transferred to the electrode vessel and NaH¹⁴CO₃ was added to give a concentration of CO₂ as for a solution equilibrated with 0.03% CO₂ in air (Fig. 2). Allowance was made as in a for CO₂ and HCO₃⁻ added with the activated enzyme and for changes of the dissociation constant with temperature (11).

(c) Assays at Subatmospheric Concentrations of CO₂ in Air. Buffer at the chosen temperature was equilibrated with CO₂-free air. Aliquots were transferred to the electrode vessel and NaH¹⁴CO₃ was added to give a concentration of CO₂ in the assay as if the solution were equilibrated with air containing 0.021% CO₂ (Fig. 2). The calculation was made using dissociation constants from Harmon and Bonner (11) and an allowance for CO₂ added to the reaction mixture with the activated enzyme.

Each reaction mixture contained 100 mM Bicine (pH 8.2), 10 mM MgCl₂, 1 mM RuBP, and 25 units of carboxic anhydride. Assays were initiated with 200 to 300 μg protein and were terminated after 30 s by adding 100 μl 4 N HCl. Aliquots of the acidified mixture were dried down and the ¹⁴C in the residue was measured by liquid scintillation counting. Oxygen concentrations were calculated from published values (18) and oxygenase activity was calculated from O₂ consumption during the assay period. CO₂ loss from solution was minimal since there was no air space above the reaction mixture.

**Protein Estimation.** The protein content of freeze-dried powders was measured according to modifications of the Lowry procedure (20, 21).

**RESULTS**

**Carboxylase and Oxygenase Activities in the Presence of 14 μM CO₂ and 0.34 mM O₂.** These concentrations were used since they are approximately the Km values of the carboxylase (5) and of the oxygenase reaction at 25°C (NP Hall, unpublished data). With both CO₂ and O₂ present together, the competitive effects of CO₂ and O₂ on the enzyme ratio are taken into account and any changes in vₒ/vₑ due to Km(CO₂), Km(O₂), Kₒ(CO₂), Kₒ(O₂), vₒ, or vₑ should be evident. The vₒ/vₑ remained constant between 5 and 25°C at 0.21 and then increased to 0.26 at 35°C (Fig. 1).

**Carboxylase and Oxygenase Activities in the Presence of CO₂ and O₂ at the Concentrations Obtained in Medium Equilibrated with Air at Each Temperature (see Fig. 2).** The results will depend on the O₂/CO₂ solubility ratio as well as on the kinetic constants mentioned above. In air-equilibrated buffer, vₒ/vₑ increased between 15 and 35°C from 0.16 to 0.35 (Fig. 1).

**Carboxylase and Oxygenase Activities in 21% O₂ Containing 210 μl/l CO₂.** The CO₂ and O₂ concentrations used in these experiments were determined by the solubility of the gases in water equilibrated with air containing 210 μl/l of CO₂. From 10 to 35°C, the ratio increased by 124% (Fig. 1). The vₒ/vₑ at 20°C was 0.3, i.e. just over three carboxylase reactions to one oxygenase.
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FIG. 3. Effect of temperature on \( P_{\text{net}} \) calculated from measurements of the enzyme activities. Carboxylase and oxygenase under subatmospheric concentrations of CO\(_2\) in air recalculated according to the equation: \( P_{\text{net}} = \nu_r - \frac{1}{2} \nu_r \) (19).

These concentrations should approximate those found in the stroma of chloroplasts of wheat leaves if the CO\(_2\) in the intercellular spaces is independent of temperature.

The measurements for soybean photosynthesis and photorespiration over the range 20 to 40\(^\circ\)C (12) were recalculated in terms of the \( \nu_r/\nu_c \) using the stoichiometry of 2 mol O\(_2\) taken up by the oxygenase per mol CO\(_2\) released in photorespiration (19). Since the original results (12) used CO\(_2\) evolution into a CO\(_2\)-free air stream in the light as a measure of photorespiration, the results probably underestimate the magnitude of CO\(_2\) products by an unknown amount (12). In recalculating the results, we have made no correction for this. The results (Fig. 1) indicate that between 20 and 35\(^\circ\)C, the ratio changed from 0.28 to 0.50 which is an increase of 79\%. The measured \( \nu_r/\nu_c \) values for the enzyme in solution equilibrated with 210 \( \mu l/l \) CO\(_2\) and 21\% (v/v) O\(_2\) increased similarly to this.

DISCUSSION

The experiments were not designed to study changes with temperature of individual kinetic constants for carboxylation and oxygenation, but to assess whether such changes were responsible for changes in the relative rates of carboxylation and oxygenation or were such as to cancel one another out and produce no effect. The advantage is that no specific model for the kinetics is assumed and measurements are made at near natural concentrations of CO\(_2\) and O\(_2\). The results above support the conclusion (16) that the differential solubility of O\(_2\) and CO\(_2\) with increasing temperature is the major factor in the O\(_2\) inhibition of photosynthesis. At constant concentrations of CO\(_2\) and O\(_2\) in solution, changes in temperature did not markedly change \( \nu_r/\nu_c \). Thus, changes in the kinetic constants for the carboxylase and oxygenase reactions, where these are defined in the usual way in terms of concentrations in solution, must have been such as to have little effect on oxygenation relative to carboxylation and cannot explain the measured increase in photorespiration and decrease in photosynthesis in C\(_3\) plants at higher temperatures (12). These findings on the isolated enzyme from wheat agree with data for wheat leaf photosynthesis where a fixed O\(_2\)/CO\(_2\) solubility ratio of 33.8 at 22\(^\circ\) or 32\(^\circ\)C gave the same percentage inhibition of photosynthesis at both temperatures (16).

The sensitivity of the \( \nu_r/\nu_c \) to changes in gas solubilities is shown by comparing the results at air levels of CO\(_2\) and O\(_2\) with those using air O\(_2\) and subatmospheric CO\(_2\). The latter results in an increase in the \( \nu_c/\nu_r \) at all temperatures. This is in keeping with the 'Bowes-Ogren Hypothesis' of the competitive interaction of CO\(_2\) and O\(_2\) on RuBP carboxylase (7). Measurements on wheat leaves have shown the sensitivity of photosynthesis to a lowering of the concentration of O\(_2\) from 21 to 3\% (v/v) which results in an increased CO\(_2\) assimilation rate and a change in the temperature optimum from 20–26\(^\circ\)C to 30–36\(^\circ\)C (15).

The Q\(_{10}\) (15–25\(^\circ\)C) obtained for photorespiration in tobacco leaves was 3.3 (27), while others suggest a Q\(_{10}\) (20–30\(^\circ\)C) of 1.4 to 1.5 for several dicotyledonous plants (12). Our data for RuBP oxygenase in subatmospheric CO\(_2\) gives an apparent Q\(_{10}\) of 1.78 between 20 and 30\(^\circ\)C. The apparent Q\(_{10}\) for the carboxylase reaction over the same temperature range and for the same O\(_2\) concentration was 1.26. This is higher than the reported 1.0 for wheat leaf photosynthesis (15). Calculation from these data for the purified enzyme of expected rates of net photosynthesis using the equation \( P_{\text{net}} = \nu_r - \frac{1}{2} \nu_r \) (19), gives a curve for photosynthesis against temperature that is similar to that for leaf photosynthesis in 21\% (v/v) O\(_2\) (15). However, optimum photosynthesis would occur between 25 and 30\(^\circ\)C instead of between 20 and 25\(^\circ\)C (15). Also, the \( P_{\text{net}} \) calculated from enzyme activities did not decline to the same extent at 35\(^\circ\)C as observed for leaves (Fig. 3). In leaves, higher temperature may cause stomata to close and thus further reduce CO\(_2\) in intercellular space. Also, the calculation did not allow for the independent increase of dark respiration with temperature that would occur in leaves.

The actual concentrations of CO\(_2\) and O\(_2\) confronting the enzyme in the chloroplast is a function of gas fluxes in and out of the leaf and may not necessarily be the same as that in the intercellular air spaces. The 'subatmospheric' conditions used in assays where the CO\(_2\) concentration was 60 to 70\% of that found in ambient air is consistent with available data for CO\(_2\) in the intercellular spaces (24). In choosing these concentrations, we have not allowed for any differential change in the solubility of CO\(_2\) in the chloroplast with temperature caused by diffusion resistance or other factors such as stomatal closure. Recent experiments with photosynthesizing chloroplasts indicate that the O\(_2\) concentration inside the chloroplast is similar to that of the external medium at an O\(_2\) concentration corresponding to air-saturated water (22). The CO\(_2\) concentration depends on the diffusion resistances and rate of photosynthesis. There are other major differences between the situation in vivo and in vitro. The concentration of RuBP carboxylase in the chloroplast is up to 240 mg/ml stromal volume and there is the unusual situation of 4 mM enzyme binding sites whereas RuBP levels are only about 0.4 mM (14). The protein concentration used in vitro was 0.4 to 0.6 mg/ml and RuBP was saturating. However, the state of activity and the rates of inactivation of the purified wheat carboxylase and the enzyme in fresh leaf extracts are similar (10). In the leaf, the enzyme is functioning under steady-state conditions and the magnitude of the steady-state is dependent on the RuBP concentration (4). The similarity in the effect of temperature on the affinity of intact leaves for CO\(_2\) and of RuBP carboxylase/oxygenase for CO\(_2\) and O\(_2\) suggests that the kinetic properties of the isolated enzyme may reflect those in vivo (17).

Therefore, while it is possible to come to the broad conclusion that the differential solubilities of O\(_2\) and CO\(_2\) constitute a major factor responsible for increased photorespiration at higher temperatures, more information concerning the actual CO\(_2\) and O\(_2\) concentrations within the chloroplast and the functioning of the enzyme in vivo is needed to further improve matching of photosynthesis and photorespiration by leaves to observed properties of purified RuBP carboxylase.
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