Oxygen Isotope Effects on the Ribulosebisphosphate Oxygenase Reaction*

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The oxygen isotope effect at the substrate O₂ on the oxygenation reaction of ribulose bisphosphate carboxylase/oxygenase from spinach is pH and metal dependent. The pH dependence between pH 7.4 and 8.9 is different with Mg²⁺ (steady decrease in this isotope effect from 1.036 to 1.030) and Mn²⁺ (minimum isotope effect of 1.028 at pH 8.0). Deuteration of the substrate (1-³H)ribulose bisphosphate) has no influence on the isotope effect. The results are interpreted as a direct participation of the metal ion in the oxygen-sensitive step, i.e. carbon-oxygen bond formation and the stabilization of the intermediates. In the overall reaction oxygen addition is a major rate-limiting step, and the observed isotope effect is probably close to the intrinsic oxygenation reaction. The basic mechanisms for carboxylation and oxygenation of ribulose bisphosphate appear to be the same.

Ribulosebisphosphate carboxylase/oxygenase (EC 4.1.1.39) catalyzes both the carboxylation and the oxygenation of D-ribulose 1,5-bisphosphate (RuBP). The oxygenation reaction, part of the photorespiration, competes with the photosynthetic CO₂ fixation. It is considered by some authors as a physiologically futile reaction, by others as a part of the plant O₂- protection system and a pathway for the formation of serine and glycolate. In C₃ plants under physiological conditions the ratio of carboxylation versus oxygenation lies between 3:1 and 4:1 (Mizioro and Lorimer, 1983). This ratio as well as the Kₘ values for the two gaseous substrates (Kₘ(CO₂) = 10–15 μM; Kₘ(O₂) = 400 μM) significantly depends on the enzyme source and the catalytical divalent metal ion (Pierce et al., 1982, 1986a).

Although most work on reaction mechanism has been done on the carboxylation reaction, there is ample evidence that also with oxygenation the enzyme activation is through carbamate formation (CO₂ as allosteric activator) and that enolization of the substrate ribulose 1,5-bisphosphate is part of the reaction sequence (Badger and Lorimer, 1976; Saver and Knowles, 1982). According to Lorimer (1981) the substrate reaction with O₂ might be through a radical mechanism producing a substrate radical and a superoxide anion prior to formation of a hydroperoxide, which then decomposes to phosphoglycolate and 3-phosphoglycerate. Electron spin resonance studies in the presence of molecular oxygen suggest that the peroxy intermediate is one of the ligands of the divalent cation (Bränden et al., 1984; Styrling and Bränden, 1985).

Earlier kinetic studies on the carboxylation reaction have been interpreted in favor of a random or an ordered mechanism of substrate addition and formation of an enzyme-CO₂-RuBP complex (Badger et al., 1980; Roeske and O'Leary, 1985). From the results of their investigations on the deuterium isotope effects on the reaction, Van Dyk and Schloss (1985) however, suggested a Theorell-Chance mechanism with CO₂ adding to the endoiol of RuBP in a bimolecular fashion. The same mechanism was also favored by Pierce et al. (1986b).

In the case of the oxygenase reaction, Badger et al. (1980) had deduced an ordered mechanism with RuBP binding first and the formation of a ternary complex prior to enolization. Later Pierce et al. (1986b) derived a Theorell-Chance mechanism also for the oxygenation based on their result that no enzyme-O₂ complex could be detected in the absence of RuBP. All these results indicate that so far the mechanism of the oxygenase reaction is not clarified totally.

The measurement of kinetic isotope effects has been very useful in the elucidation of relative velocities of different steps of the carboxylation reaction (Roeske and O'Leary, 1985; Van Dyk and Schloss, 1986). So far, this approach has only recently been made for the oxygenation reaction. The studies of Guy et al. (1987) on the oxygen isotope effect of the RuBP oxygenase were mainly orientated at the importance of this reaction for the oxygen isotope fractionation during photorespiration in plants. In this work, we investigate the influence of pH, metal, and deuteration of the substrate RuBP on the oxygen isotope effect on the RuBP carboxylase/oxygenase reaction and discuss the results in context with the mechanism of the reaction.

EXPERIMENTAL PROCEDURES

Materials and Enzyme Preparation—RuBP (Ba²⁺ salt) was prepared as described by Hoecker et al. (1958) and stored at –20 °C. [3-³H]RuBP (Li⁺ salt) was a gift from Dr. John Schloss, Du Pont de Nemours, Wilmington, DE. Most organic compounds and enzymes were from Sigma Chemie GmbH, München, Federal Republic of Germany (FRG), Sephorose 4B and Sephadex G-25 were from Pharmacia GmbH, Freiburg, FRG, Tris was from E. Merck, Darmstadt, FRG. Ribulosebisphosphate carboxylase/oxygenase was isolated from fresh, deveined spinach leaves by a modification of the procedure of McCurry et al. (1982). After fractionated precipitation of the crude extract with ammonium sulfate, the enzyme solution was desalted in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.


The abbreviation used is: RuBP, D-ribulose 1,5-bisphosphate.
genase was diluted and activated (15 min, 22°C) in 0.1 M Tris buffer pH 8.2, with 10 mM NaHCO3, 1 mM dithiothreitol, 10 mM MgCl2, or 5 mM MnCl2. All solutions for the oxygenase reaction were prepared with CO2-free water. The oxygenase activity of the enzyme (at 22 and 26°C) was determined with a Clark oxygen electrode (Beckman, München, FRG) according to Badger and Lorimer (1976). The reaction medium contained in a total volume of 1 ml of 0.15 M Tris buffer of various pH values (pH 7.0–9.0), 0.6 mM RuBP, 20 mM MgCl2 or 0.5 mM MnCl2, equilibrated with CO2-free air. The reaction was started by adding the solution of the activated enzyme (0.1 ml), and the decrease of the oxygen concentration was recorded. The assay for the carboxylase activity was according to the method of Lilley and Walker (1974).

**Oxygen Isotope Effects—**A special reaction vessel (Fig. 1) for the determination of oxygen isotope effects was used. 55 ml of CO2-free 0.1 M Tris (different pH values), 0.6 mM RuBP (or [3H]RuBP), 20 mM MgCl2 or 0.5 mM MnCl2, respectively, were filled into compartments R1 and R2 and saturated with oxygen. Then, by motion of the piston in R2 compartment R3 was also filled with the solution, and stopcock S3 was closed. 1.4 ml of solution in R3 were replaced by 1.4 ml of enzyme-free activation medium by means of a syringe, and then stopcock S2 was closed. The reaction was started by injection of the activated enzyme (1.5 units in 1.4 ml of activation medium) through the rubber septum I. The progress of the reaction at 22 or 26°C was controlled by means of an oxygen electrode. After partial turnover (given as fraction of reaction) the main stopcock S3 was opened, and the solution from R3 was poured through C into an evacuated cold trap at −196°C. The dissolved oxygen was quantitatively collected (three freeze/thaw cycles) into a sample tube by means of a Toeppler pump. Thereafter, the oxygen from the medium in the reference compartment R2 was collected in the same way.

The 18O/16O ratio of the sample oxygen (R2) was measured relative to that from the reference (R2) on a VG Micromass 903 isotope ratio mass spectrometer. The kinetic isotope effects were calculated according to Equation 1 (O’Leary, 1980).

$$k_{16}/k_{18} = \log(1 - f) / \log(1 - f)_{R2}/R2$$

**Carbon Isotope Effect of C-3 of RuBP—**The enzyme was activated as described above, however, 0.25 mM NaHCO3 was used. The reaction proceeded in the same vessel and under the same conditions as described for the determination of the oxygen isotope effect (Mg2+ as catalytic metal ion). For experiments with partial turnover the RuBP concentration was 5 mM. The fraction of reaction was measured in aliquots by enzymatic determination of the 3-phosphoglycerate (Czok, 1970). The solutions (partial and complete turnover) were immediately cooled in liquid nitrogen, then lyophilized, and the residue, containing the 3-phosphoglycerate formed, was dissolved in 3 ml of 1 N HCl and centrifuged. To the supernatant 100 mg of BaCl2 were added and the solution was neutralized with KOH. The precipitate was isolated by centrifugation, washed with ethanol, and redisolved in 5 ml of 0.1 N HCl. The excess of Ba2+ was precipitated as BaSO4 (addition of Na2SO4) and separated by centrifugation. The neutralized solution was chromatographed on a Dowex 1 × 8 column (28 × 0.5 cm) with a 0–0.1 N HCl linear gradient. The fractions containing the 3-phosphoglycerate were combined, and the organic acid was decarboxylated enzymatically according to Roeseke and O’Leary (1984). The CO2, which corresponds to C-3 of RuBP, was isolated, and isotopic analysis and calculation of the isotope effect were done analogous to the oxygen samples.

**RESULTS**

**Oxygenase and Carboxylase Activity of the Enzyme—**Ribulosebiphosphate carboxylase/oxygenase needs HCO3 and a divalent cation for activation in catalysis of the oxygenation reaction. As Mn2+ significantly decreases the Kcat value for O2 as compared with Mg2+ (Christeller and Laing, 1979), the concentrations of the two cations in the experiments were fixed in order to attain about the same specific oxygenase activity. Under otherwise identical conditions at pH 8.2, 0.110 units/mg protein were measured in 20 mM Mg2+ and 0.156 units/mg protein in 0.5 mM Mn2+, which is in line with earlier results of Wildner and Henkel (1978).

In order to favor the oxygenase activity, the incubation was performed in an O2-saturated, CO2-depleted medium. With 1100 μM O2 and 10 μM CO2 (pH 8.0; 22°C), the ratio of phosphoglycolate formed to O2 consumed was 0.98 (theoretically 1.0, Harris and Stern, 1978), and, as calculated from the amount of phosphoglycolate formed, the ratio of oxygenase to carboxylase activity was 4.8:1 (under physiological conditions 0.25–0.33:1, Miziorko and Lorimer, 1983). With an Mn2+- and CO2-activated enzyme, the oxygenase activity was determined depending on the pH value in presence of 0.5 mM Mn2+ (26°C). The bell-shaped curve had an optimum at pH 8.2 and was similar to the curve obtained by Andrews et al. (1975) with a pH optimum at 8.5 (25 mM Mg2+).

**Oxygen Isotope Effects—**For these measurements the incubation media had been saturated with O2 (known isotopic composition, [18O/16O] = R0). After partial turnover of the substrate O2 (30–60%, monitored with an oxygen-sensitive electrode) the isotopic composition of the remaining dissolved oxygen gas was measured ([18O/16O] = R0), and the isotope effect of the reaction was calculated according to Equation 1. The results obtained at different pH values (7.4–8.9) and temperatures and with Mg2+ and Mn2+, respectively, are shown in Table I and Fig. 2.

In the presence of Mg2+, the isotope effects at 26°C show a slight steady decrease with increasing pH, while with Mn2+ as catalytic metal ion the values for the isotope effects go through a minimum at pH 8.0. With Mg2+ all measured values for the isotope effects at 22°C are below those at 26°C. A conformational change of the enzyme could be discussed as the possible reason. As a matter of fact an Arrhenius plot of the oxygenase activity shows a break at 15°C and not in the range in question here (Badger et al., 1977). Therefore, a final interpretation of our result cannot be given. A deuteration substitution at C-3 has practically no influence on the isotope effect.

**Carbon Isotope Effect at C-3 of RuBP—**The phosphoglycerate formed by the RuBP oxygenase reaction after 100% and 12% turnover was isolated and enzymatically decarboxylated.
The standard 13C value is defined as:

$$\delta^{13}C(\%o) = \left[ \frac{^{13}C/^{12}C}_{\text{sample}} / \frac{^{13}C/^{12}C}_{\text{standard}} - 1 \right] \times 1000$$

The standard is carbon dioxide from PeeDee Belemnite isotopic standard limestone (Craig, 1957).

Fig. 2. The pH and metal dependence of the oxygen isotope effect of the ribulosebisphosphate oxygenase reaction at 26°C. Mg2+ (A), Mn2+ (O).

The CO2 isolated had a δ13C-value² of −13.84‰ (100% turnover, mean value of three experiments) and −19.28‰ (12% turnover, mean value of four experiments), respectively. This corresponds to a kinetic isotope effect at C-3 of RuBP of $k^{13}/k^{18} = 1.006 \pm 0.003$. Our value is very close to the one found by Roeske and O’Leary (1984) for the same carbon atom in the carboxylation reaction (1.0082).

DISCUSSION

The carbon isotope effect at CO2 on the RuBP carboxylase reaction has been measured several times (for a review see O’Leary, 1981), and also the influence of the metal cofactor and its concentration, ionic strength, and pH value on the isotope effect (Winkler et al., 1982) have been studied. The generally accepted value of 1.029 under physiological conditions (Roeske and O’Leary, 1984) indicates that carboxylation is a partially rate determining step of the reaction.

In the case of the oxygenase reaction, the values reported here (near 1.03) indicate likewise that in this reaction oxygenation is partially rate limiting. It is generally accepted that the principal mechanism for the oxygenase reaction comprises the binding and enolization of RuBP and the formation of a peroxo intermediate prior to carbon-carbon bond cleavage (Scheme 1; $E = \text{enzyme}$). So far, however, it is not unambiguously decided whether the addition of O2 occurs before (upper path) or after enolization (lower path). Badger et al. (1980) postulated an ordered binding mechanism and formation of a ternary enzyme-RuBP-O2 complex. Studies by Pierce et al. (1986a) on the other hand revealed no evidence for a binding site for O2, which makes a Theorell-Chance type of mechanism, analogous to the mechanism proposed by Van Dyk and Schloss (1986) for the carboxylase reaction, more likely. Independently, in both mechanisms, the only oxygen isotope-sensitive step on O2 should be the one connected to the rate constants $k_7$ and $k_8$.

Accordingly, the general equation for the observed isotope effect ($k^{18}/k^{13}$ in Table I) is

$$k^{18}/k^{13} = \frac{k_{13}/k_{18} + c_f + c_r \cdot K_{eq}}{1 + c_f + c_r}$$

where $k_{13}/k_{18}$ is the intrinsic isotope effect, $c_f$ the forward commitment, $c_r$ the reverse commitment, and $K_{eq}$ the equilibrium isotope effect on the reversible isotope-sensitive step (Cleland, 1982). The explicit forms of Equation 2 applied to the two mechanisms in question (Scheme 1) are Equation 3 for the upper path and 4 for the lower path, respectively.

$$k^{18}/k^{13} = \frac{k_8/k_7 \cdot + k_7/k_8 (1 + k_8/k_7 (1 + k_7/k_8)) + k_7/k_8 \cdot k_1/k_8 \cdot + k_8/k_1 \cdot k_7/k_8}{1 + k_8/k_7 (1 + k_7/k_8 (1 + k_8/k_7)) + k_7/k_8}$$

$$k^{18}/k^{13} = \frac{k_7/k_8 \cdot + k_8/k_7 \cdot + k_7/k_8 \cdot k_1/k_8 \cdot + k_8/k_1 \cdot k_7/k_8}{1 + k_8/k_7}$$

We have measured the oxygen isotope effect with RuBP and RuBP deuterated in position C-3 and found no difference within the experimental error. The very deuterium isotope effect of the oxygenase reaction has not been determined so far, however, Van Dyk and Schloss (1986) did the corresponding experiments on the carboxylase reaction (enzyme from spinach). Their data provide strong evidence that the enolization step is deuterium-sensitive and one of the rate-limiting steps. Assuming the same for the oxygenase reaction, the fact that the oxygen isotope effect is not affected by 3H substitution of the substrate is only consistent with an expression according to Equation 4 (no effect by a decrease in $k_8$). This is in line with O2 reacting only after enolization without formation of a ternary complex (lower path of Scheme 1). The identical reaction sequence of binding and enolization of RuBP for carboxylase and oxygenase reaction would also be
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The formation of an intermediary hydroperoxide (described by $k_7/k_7'$ in Equation 4) would be the oxygen isotope-sensitive step. This reaction most probably implies activation of $O_2$ with spin inversion (Lorimer, 1981) and formation of the C-O bond. Its intrinsic isotope effect can only be estimated, since to our knowledge no directly comparable enzymic or model reactions have been studied. As to other examples, the measured oxygen isotope effect of the cytochrome oxidase reaction is 1.013 (Feldman et al., 1959), and for the formation of a carbon-oxygen bond by addition of water to a C-C double bond (fumarase reaction), Blanchard and Cleland (1980) calculated an intrinsic oxygen isotope effect between 1.041 and 1.045. For the RuBP oxygenase reaction, we assume that the equilibrium isotope effect $(k_i/k_i')/(k_a/k_a')$ is close to unity.

The value of the ratio $k_a/k_b$ can be expected to be rather small ($k_b \ll 1$), as comparable chemical reactions, the peroxide formation of dicarbonyl compounds are practically irreversible (Frankvoort, 1978). Accordingly, the value for the observed isotope effect $k^{16}/k^{18}$ (maximum value 1.036) should be close to the intrinsic isotope effect $k_7/k_7'$. We therefore deduce that oxygenation is the major rate-limiting step in the RuBP oxygenase reaction. This conclusion is corroborated by the size of the carbon isotope effect at C-3 of RuBP. Isotope-sensitive steps concerning this carbon are enolization ($k_6$) and C-C bond splitting ($k_8$). For the latter reaction step, a high intrinsic isotope effect can be expected (cf. Roeske and O'Leary, 1984, for the carboxylase reaction). As the observed isotope effect, however, is rather small (1.006), the corresponding commitments must be high. This means that C-C bond cleavage and most probably enolization also are faster (high values for $k_s/k_2$ and $k_6/k_8$) and less rate limiting than the oxygenation step ($k_7$).

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3 H. M. O'Leary, personal communication.
The oxygen isotope effect varies with temperature, pH, and catalytic cation, indicating changes in the ratio $k_A/k_B$. The most remarkable phenomenon is the difference in the pH dependence with $Mg^{2+}$ and $Mn^{2+}$, respectively. A function discussed for the metal ion is its participation in the enediel formation and stabilization. Not only from our results on the different influence of $Mg^{2+}$ and $Mn^{2+}$, we postulate, in addition, a direct participation in the oxygenation step (Scheme 2). ESR studies by Bränden and co-workers (Bränden et al., 1984; Styring and Bränden, 1985) and Miziorko and Sealy (1984) on RuBP carboxylase/oxygenase indicate a 6- or 5-coordinated metal complex at the active site, including groups of the substrate, intermediate or product, and $H_2O$ molecules. Direct coordination between the hydroperoxide anion and the metal as in complex II is in full agreement with spectroscopic data for the enzyme under oxygenase favoring conditions (Bränden et al., 1984).

The fact that the isotope effects are different with $Mg^{2+}$ and $Mn^{2+}$, respectively, is in line with the known differences of these two ions as activator and catalyst in the oxygenase reaction. $Mn^{2+}$ is a better complexing agent than $Mg^{2+}$, and its solubility product with $OH^-$ is smaller by more than two orders of magnitude. This may have for consequence shifts in the binding behavior of different groups as ligands, and hence differences in the transition state. As already mentioned, the stabilization of complex II is metal dependent, and the ion influences the reaction rates, causing a change in the ratio $k_A/k_B$. Furthermore, these rates are pH dependent. The increase above pH 8 in the observed isotope effect with $Mn^{2+}$ present might be due to a decrease in the solubility of $Mn^{2+}$ complexes and a change in the ligands of the enzyme complex.

Differences in the isotope effect depending on the catalytic ion had also been found for the RuBP carboxylase reaction (Estep et al., 1978; Winkler et al., 1982). Probably these differences in both carboxylation and oxygenation are related to the fact that under physiological conditions $Mg^{2+}$ enhances carboxylation, whereas $Mn^{2+}$ favors the oxygenase reaction of the enzyme.

In conclusion, our results strongly indicate an active role of the metal on the enediel intermediate stabilization and reaction with $O_2$. The oxygenation seems to proceed principally by the same Theorell-Chance type of mechanism as the carboxylation with oxygen reacting directly with the enzyme-enediel complex.

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REFERENCES


