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An Intermediate Formed by the Cu^{2+}-Activated Ribulose-1,5-bisphosphate Carboxylase/Oxygenase in the Presence of Ribulose 1,5-Bisphosphate and O_2†

Rolf Brändén,* Thomas Nilsson, and Stenbjörn Styring

ABSTRACT: When ribulose 1,5-bisphosphate (RuBP) is added to the Cu^{2+}-incubated RuBP carboxylase/oxygenase, three different enzyme–Cu^{2+} complexes are observed by EPR spectroscopy. All three complexes have similar but distinct EPR spectra with very narrow hyperfine lines, which indicate that only oxygen atoms are coordinated to the Cu^{2+} ion. One of them is a rapidly formed, transient, oxygen-dependent enzyme–Cu^{2+} complex whose slow decomposition is correlated to the production of phosphoglycolate and 3-phosphoglyceric acid. By use of $^{17}O_2$, it was proved that one of the atoms coordinated to Cu^{2+} in this complex originated from molecular oxygen. A hydroperoxide intermediate formed in the RuBP oxygenase reaction may provide one of the oxygen ligands in this transient and oxygen-dependent enzyme–Cu^{2+} complex. Effects on the EPR spectra on substituting H_2O for D_2O in the assay indicate that water is another ligand to the metal in these complexes.

Growing knowledge on the RuBP[1] carboxylase/oxygenase mechanism has been accumulated during the last decade, and now a reasonable model for the carboxylation of RuBP exists (Miziorko & Lorimer, 1983). This model includes the formation of an enediol form of RuBP that is involved in the nucleophilic attack on CO_2, yielding a transient six-carbon intermediate. A water molecule rapidly reacts at the C-3 position of the intermediate, and the products of the carboxylase reaction, two molecules of 3-PGA, are formed.

A mechanism analogous to that proposed for the carboxylase reaction has been offered to explain the oxygenation of RuBP (Lorimer, 1981). Here molecular oxygen reacts with the enediol of RuBP, and an intermediate, 2-peroxy-3-oxopentitol 1,5-bisphosphate, is suggested to be formed. The attack of a hydroxyl ion on the C-3 carbon of the intermediate initiates its cleavage to the products of the oxygenase reaction, phosphoglycolate and 3-PGA.

The six-carbon intermediate in the carboxylase reaction has been experimentally verified (Schloss & Lorimer, 1982), but the existence of a hydroperoxide intermediate in the oxygenase reaction has, so far, not been demonstrated. Furthermore, no really acceptable explanation has yet been offered for the important problem of how molecular oxygen can form a hydroperoxide in the absence of any enzyme cofactor.

A divalent metal ion is required for the activation of the enzyme, but a role for the metal in catalysis has also been suggested. Strong evidence for such an involvement comes from ^{13}C NMR data (Miziorko & Mildvan, 1974), which indicate that the metal (Mn^{2+}) and the catalytic CO_2 are close enough to allow interaction. The drastic drop of K_i for O_2 for the Mn^{2+}-activated enzyme compared to that for the Mg^{2+}-activated one (Christeller, 1981) as well the F^- inhibition of the enzyme (Nilsson & Brändén, 1983) also supports this idea.

Further elucidation of the involvement of the metal ion in catalysis of RuBP carboxylase/oxygenase may be facilitated by the use of spectroscopic studies on suitable metal probes. Electron paramagnetic resonance (EPR) studies of the Cu^{2+}-incubated enzyme in the presence of HCO_3^- indicate that Cu^{2+} binds at the same site as other activating metal ions (Brändén et al., 1984b). An enzyme–Cu^{2+}–RuBP complex with a characteristic EPR spectrum was formed in the presence of RuBP, which implied the possibility of studying the role of metal ion in catalysis in more detail.

Little is known about the metal binding site. The carbamate formed in the activating process is stabilized by the metal (Lorimer & Miziorko, 1980) and a direct coordination of the

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[1] Abbreviations: EPR, electron paramagnetic resonance; RuBP, ribulose 1,5-bisphosphate; HEPPS, *N*-(2-hydroxyethyl)piperazine-*N'*-propanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; CABP, 2-carboxyarabinitol 1,5-bisphosphate; 3-PGA, 3-phosphoglyceric acid.

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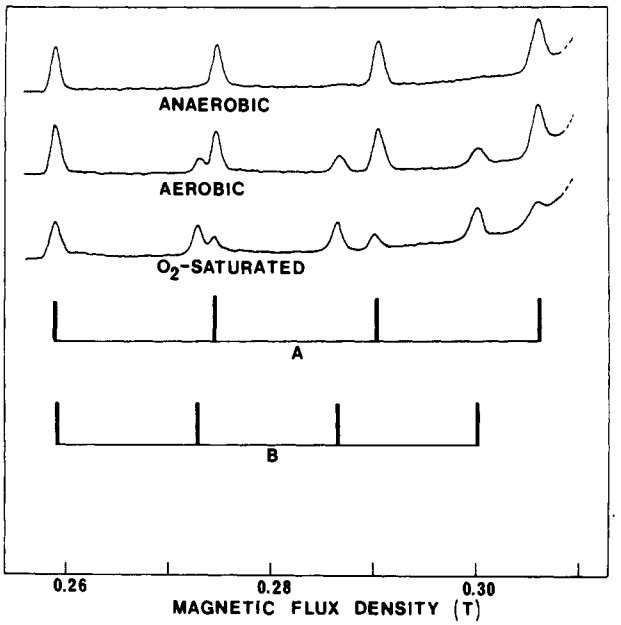

FIGURE 1: Effect of O_2 on the EPR spectra of Cu^{2+}-incubated RuBP carboxylase/oxygenase obtained in the presence of excess RuBP. Anaerobic condition: to 250 μL of anaerobic enzyme solution was added 15 μL of $NaHCO_3$ (0.5 M), followed by 10 μL of $^{63}CuCl_2$ (12.5 mM) and 2 min thereafter 25 μL of RuBP (0.1 M). All added solutions were made anaerobic by N_2 flushing for 5–10 min. Aerobic condition: 250 μL of an enzyme solution saturated with air was treated as the anaerobic solution except that all added solutions were saturated with air. O_2-saturated condition: 250 μL of an enzyme solution was saturated with O_2 and treated as the anaerobic enzyme solution except that all added solutions were saturated with O_2. All three samples were frozen in EPR tubes at liquid nitrogen 15 min after the addition of RuBP, and all additions were made to an enzyme solution (41 mg/mL) in 50 mM HEPPS buffer at pH 8.0 and 25 °C. The spectrometer gain was 8×10^4 and the modulation amplitude was 1 mT. A and B indicate the EPR hyperfine lines of the two different complexes (complexes A and B) that were formed.

carbamate to the metal has therefore been suggested but this has, so far, not been experimentally verified. In the preceding paper (Brändén et al., 1984b) we showed that only oxygen atoms are directly coordinated to the Cu^{2+} ion in the enzyme–Cu^{2+}–RuBP complex. Some of the carboxylic side chains near lysine-201 (which forms the carbamate with the activator CO_2) may provide the oxygen ligands for this complex. The other oxygen ligands may be derived from the substrate or from water. Here we show that an intermediate is formed in the reaction between RuBP and O_2 in the presence of the Cu^{2+}-incubated enzyme. This intermediate has an oxygen atom, originating from molecular oxygen, directly coordinated to the metal, and its decomposition is correlated to the formation of phosphoglycolate and 3-PGA. We therefore suggest that this oxygen intermediate is the proposed hydroperoxide intermediate formed in the RuBP oxygenase reaction.

We also present results obtained on substituting H_2O for D_2O, which suggest that at least one water molecule is coordinated to the metal in the three different enzyme–Cu^{2+} complexes formed when RuBP is added to the Cu^{2+}-incubated enzyme.

Preliminary accounts of some of the results have been given at recent conferences (Brändén et al., 1984a; Styring et al., 1983).

Materials and Methods

The $^{17}O_2$-enriched O_2 (91.8%) was purchased from Miles Laboratories, Ltd. All other materials and methods were the same as described previously (Brändén et al., 1984a).

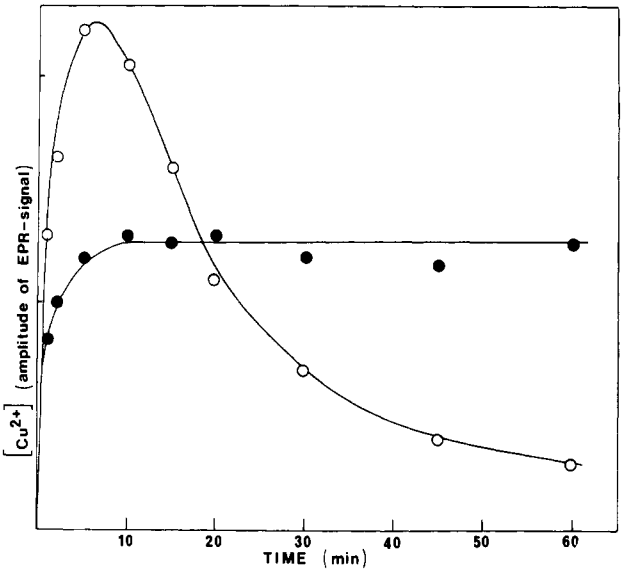

FIGURE 2: Time course for the formation of the two EPR signals formed under O_2-saturated conditions and in the presence of excess RuBP. To 3 mL of an O_2-saturated enzyme solution (33 mg/mL in 50 mM HEPPS buffer at pH 8.0 and 25 °C) was added 75 μL of $NaHCO_3$ (1 M), followed by 75 μL of $^{63}CuCl_2$ (12.5 mM) and 2 min thereafter 100 μL of RuBP (0.25 M). At different times after the addition of RuBP samples were taken and frozen in EPR tubes at liquid nitrogen. During the whole reaction the enzyme solution was saturated with O_2. The amounts of complexes A and B that were formed were obtained from the amplitudes of the hyperfine lines at 0.290 (●) and 0.286 T (○), respectively.

Results

Effects of RuBP and O_2 on the EPR Spectra of the Cu^{2+}-Incubated RuBP Carboxylase/Oxygenase. Three different enzyme–Cu^{2+} complexes could be observed when RuBP was added to Cu^{2+}-incubated RuBP carboxylase/oxygenase at pH 8.0 in the presence of 25 mM HCO_3^-. Crucial for the type of complex formed were the concentrations of RuBP and O_2.

In the presence of excess RuBP and under anaerobic conditions the enzyme–Cu^{2+}–RuBP complex (complex A) described in the preceding paper was obtained (Figure 1). This complex was rapidly formed (Figure 2) and was found to be stable for several hours at room temperature (Brändén et al., 1984b).

When RuBP was added to an aerobic or O_2-saturated enzyme solution, another enzyme–Cu^{2+} complex (complex B) was formed besides complex A (Figure 1). The narrow EPR hyperfine lines of the new complex indicate that only oxygen atoms are coordinated to the Cu^{2+} ion just as suggested for complex A (Brändén et al., 1984b). The amount of complex B that was formed was dependent on the concentration of O_2 as the EPR signal intensity increased at higher concentrations of O_2 (Figure 1).

The rates for the formation of complexes A and B are approximately equal (Figure 2). However, complex B is unstable, in contrast to complex A, and decomposes with a half-time of about 20 min.

When stoichiometric amounts of RuBP were added and the sample was frozen after 25 s, only complex B was observed (Figure 3). If the mixture was frozen after longer times, the amount of complex B was decreased and a new complex (C) with the EPR spectrum shown in Figure 3 appeared.

An enzyme–Cu^{2+}–D-3-PGA complex formed by the addition of stoichiometric amounts of D-3-PGA to the Cu^{2+}-incubated enzyme, in the absence of RuBP, gave an EPR spectrum identical with that obtained for complex C (Figure 3). On

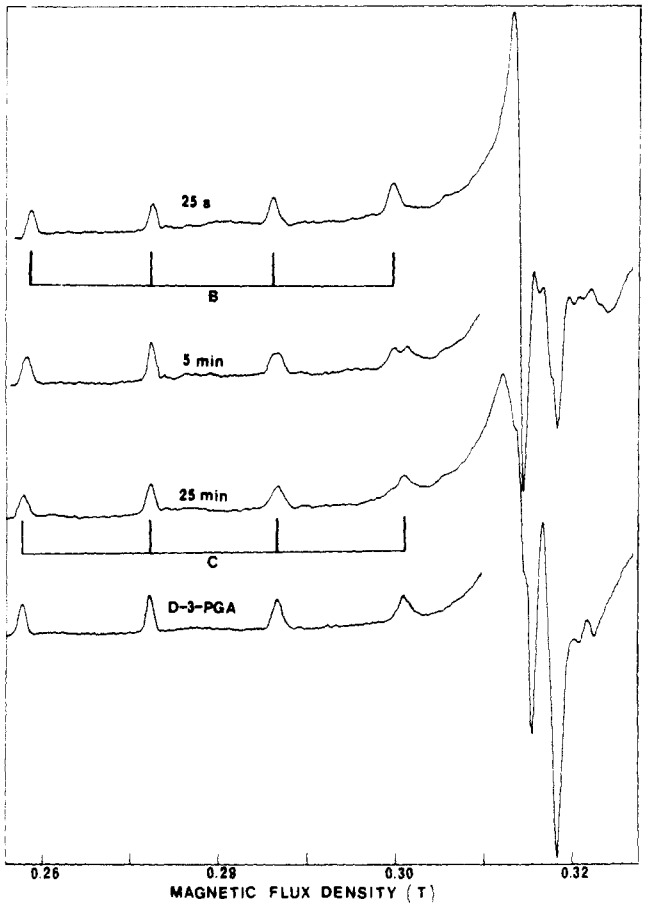

FIGURE 3: EPR spectra obtained when stoichiometric amounts of RuBP or D-3-PGA were added to Cu^{2+}-incubated RuBP carboxylase/oxygenase under aerobic conditions. To 800 μL of enzyme (30 mg/mL in 50 mM HEPPS buffer at pH 8.0 and 25 °C, containing 25 mM HCO_3^-) was added 20 μL of $^{63}CuCl_2$ (12.5 mM). After 2 min, 20 μL of RuBP (15 mM) was added, and samples were taken and frozen in EPR tubes at liquid nitrogen at 25 s, 5 min, and 25 min. D-3-PGA spectrum: to 400 μL of the same enzyme solution as above was added 10 μL of $^{63}CuCl_2$ (12.5 mM). After 2 min 10 μL of D-3-PGA (15 mM) was added, and the solution was frozen in an EPR tube at liquid nitrogen after 15 min at room temperature. The spectrometer gain was 1×10^5 and the modulation amplitude was 0.5 mT. The hyperfine lines of the two complexes are indicated as B and C for complexes B and C, respectively.

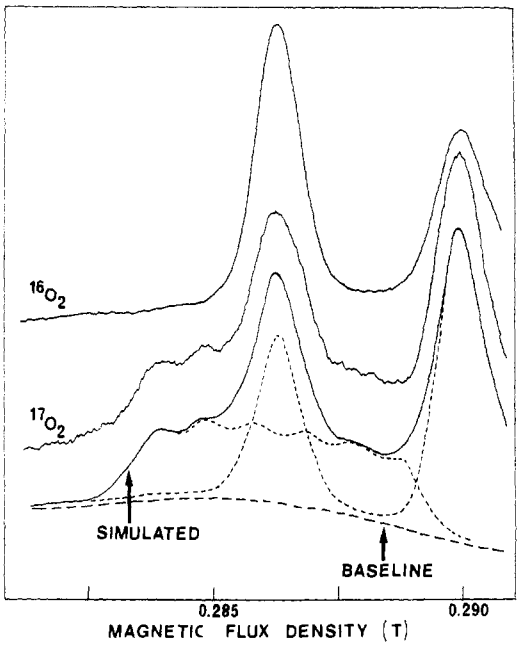

FIGURE 4: Enlarged part of the EPR spectra obtained when RuBP was added to Cu^{2+}-incubated RuBP carboxylase under $^{16}O_2$- or $^{17}O_2$-saturated conditions. To 400 μL of enzyme (33 mg/mL in 50 mM HEPPS buffer at pH 8.0 and saturated with $^{16}O_2$ or $^{17}O_2$) were added 25 μL of $NaHCO_3$ (1 M) and 10 μL of $^{63}CuCl_2$ (20 mM). After 5 min, 15 μL of RuBP (0.25 M) was added and the solution was kept under O_2-saturated conditions for 10 min before it was frozen in an EPR tube at liquid nitrogen. The simulation is based on the assumption that only one ^{17}O atom ($I = {}^5/_2$) interacts with the electron spin of Cu^{2+}. A superhyperfine splitting constant of 0.96 mT and an assay containing 70% $^{17}O_2$ and 30% $^{16}O_2$ were also assumed. The spectrometer gains were 3.2×10^5 and 1×10^6 for the EPR spectra obtained in the presence of $^{16}O_2$ and $^{17}O_2$, respectively. The modulation amplitude was 0.5 mT.

Table I: Effect of D_2O on the EPR Hyperfine Lines of Complexes A, B, and C[a]

type of complex	position of hyperfine line (T)	line width in H_2O (mT)	difference in line widths ($H_2O - D_2O$)
complex A	0.2905	1.00	0.15
complex B	0.2867	0.90	0.07
complex C	0.2733	0.79	0.15

[a] The line widths at half-amplitude are given.

the other hand, the addition of phosphoglycolate, under the same conditions, gave an EPR spectrum with broad hyperfine lines similar to that obtained in the absence of RuBP (Brändén et al., 1984a).

Effect of $^{17}O_2$. $^{17}O_2$ was used to decide whether oxygen atoms, originating from the dioxygen molecule, were directly coordinated to the Cu^{2+} ion in complex B. When an excess of RuBP was added to Cu^{2+}-incubated enzyme in $^{17}O_2$-saturated buffer, a broadening was observed for the EPR hyperfine lines of complex B (Figure 4). The experimental EPR spectrum can be simulated if it is assumed that the nuclear spin of one ^{17}O atom ($I = {}^5/_2$) interacts with the electron spin of Cu^{2+} (Figure 4). One atom of oxygen, originating from molecular oxygen, must therefore be directly coordinated to the Cu^{2+} ion of complex B. A certain amount of the hyperfine lines of complex B was not broadened by $^{17}O_2$ as seen in Figure 4. This is due to contaminating $^{16}O_2$ in the sample. It should also be noted that two of the individual superhyperfine lines are resolved at the low-field part of the spectrum.

A certain amount of the added copper is known to be bound to the enzyme as Cu^+ (Brändén et al., 1984b). H_2O_2 may therefore be formed in a reaction between O_2 and Cu^+ ions and give rise to the formation of complex B. Experiments were therefore performed in the presence of H_2O_2. H_2O_2 (0.5–2.0 mM) was added either at the same time as RuBP or to already formed complexes A, B, and C. No effect of H_2O_2 could be observed on any of the three complexes formed. Neither did the amount of complex B increase in the presence of H_2O_2. H_2O_2 is therefore not likely to be the species giving rise to complex B and the effect of $^{17}O_2$ on the EPR spectrum of this complex.

Effect of D_2O. Some experiments were performed with enzyme dissolved in buffer made of D_2O. The line widths of the EPR hyperfine lines of the three complexes made under this condition were compared to the line widths obtained in the presence of H_2O. An effect due to the different magnetic moments of ^1H and ^2H may be observed if exchangeable protons are in the coordination sphere of Cu^{2+}. Figure 5 and Table I show that D_2O has a narrowing effect on the three complexes. For complexes A and C the same narrowing (0.15 mT) was obtained, while for complex B a narrowing of 0.07 mT was observed. The narrowing effect on complex B was thus only half of that observed for complexes A and C, indicating that only half the number of protons interacts with

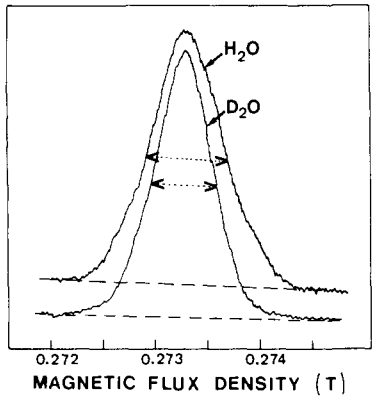

FIGURE 5: Effect of D_2O on a hyperfine line in the EPR spectrum of complex C. To 400 μL of enzyme (40 mg/mL in 50 mM HEPPS buffer in H_2O or D_2O at pH 8.0, containing 25 mM $NaHCO_3$) were added 10 μL of $^{63}CuCl_2$ (12.5 mM) and 2 min thereafter 10 μL of D-3-PGA (20 mM). After 10 min the sample was frozen in an EPR tube at liquid nitrogen. The spectrometer gain was 3.2×10^5 and the modulation amplitude was 0.5 mT.

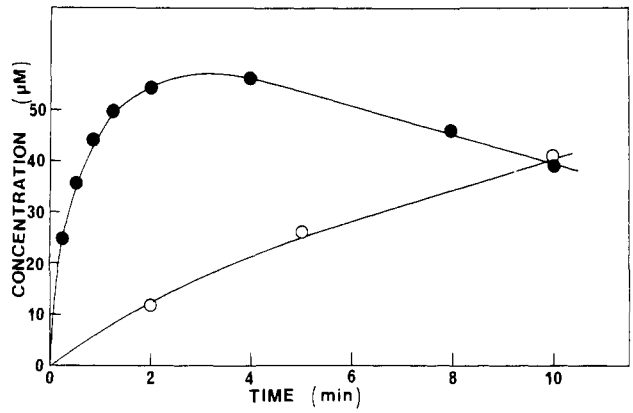

FIGURE 6: Kinetics of the oxygen-dependent EPR signal (complex B) and of phosphoglycolate production. To 4.0 mL of enzyme (13.5 mg/mL in 50 mM HEPPS buffer at pH 8.0, containing 50 mM $NaHCO_3$) was added 30 μL of $^{63}CuCl_2$ (20 mM). After 2 min, 100 μL of RuBP (9.5 mM) was added. Aliquots were taken at different times and frozen in EPR tubes at liquid nitrogen. Some aliquots were quenched and analyzed for phosphorus compounds. (●) Concentration of complex B; (○) concentration of phosphoglycolate.

Cu^{2+} in complex B as compared to complexes A and C.

Phosphoglycolate Production. RuBP was added to Cu^{2+}-incubated enzyme and aliquots were taken at different times. Some aliquots were frozen in EPR tubes at liquid nitrogen, and some were quenched in $HClO_4$ for analysis of phosphorus compounds by high-pressure liquid chromatography as described in Brändén et al. (1984b). Figure 6 shows the kinetics of the formation and decay of complex B and of phosphoglycolate production. It is clearly seen that complex B is formed much faster than phosphoglycolate. The maximum amount of complex B was reached after 2 min, and at this time only a minor amount of phosphoglycolate had been formed. The rate of decomposition of complex B, however, seems to be correlated to the production of phosphoglycolate. The initial rate of phosphoglycolate formation is ca. 0.5 nmol of phosphoglycolate formed min^{-1} mg^{-1}, which is about 10% of the RuBP carboxylase activity obtained under the same condition (Brändén et al., 1984b).

The formation of 3-PGA was faster than the phosphoglycolate production, due to the carboxylase reaction. Control experiments were done to test if phosphoglycolate was produced in the absence of Cu^{2+} under otherwise identical conditions. Less than 10% of that seen in Figure 6 was produced in the absence of Cu^{2+}.

Table II: EPR Parameters of RuBP Carboxylase/Oxygenase–Cu^{2+} Complexes (in 50 mM HEPPS Buffer at pH 8.0 Containing 25 mM HCO_3^-)

type of complex	$g_\parallel$	$g_\perp$	$A_\parallel$ (mT)	$A_\perp$ (mT)	line width at half-amplitude[b] (mT)
enzyme–Cu^{2+}	2.28	2.07	15.7	nd[a]	3.3
complex A	2.30	2.06	15.8	2.39	1.0
complex B	2.32	nd	13.5	nd	0.9
complex C	2.31	nd	14.3	nd	0.8
enzyme–Cu^{2+}–CABP	2.41	2.08	10.3	nd	1.0
enzyme–Cu^{2+}–3-PGA	2.31	nd	14.3	nd	0.8

[a] nd = not determined. [b] Line width of the hyperfine line at lowest magnetic flux density.

Discussion

Three different enzyme–Cu^{2+} complexes were observed when RuBP was added to the Cu^{2+}-incubated RuBP carboxylase/oxygenase. If the same kind of complexes are formed in the presence of other activating metal ions, a characterization of these complexes must be of great importance for an understanding of the RuBP carboxylase/oxygenase mechanism.

The following findings strongly support our suggestion that these three enzyme–Cu^{2+} complexes have only oxygen atoms coordinated to the metal ion. The narrow EPR hyperfine lines observed for all three complexes (see Table II) are not compatible with nitrogen atom(s) coordinated to the Cu^{2+} ion (Brändén et al., 1984b). The model compound Cu^{2+}–acetyl acetonate (Vänngård, 1972), with only oxygen atoms as ligands to Cu^{2+}, has a similar EPR spectrum to those of complexes A, B, and C. Narrow EPR hyperfine lines have been observed for Cu^{2+}-incubated enolase (Dickinson et al., 1980) and pyrophosphatase (B. Cooperman, personal communication), and here oxygen atoms are suggested to be the only ligands to the Cu^{2+} ion.

Oxygen atoms coordinated to the metal must originate from one of the following sources: H_2O; oxygen atoms of enzyme residues; oxygen atoms from substrate or product molecules. For complex A we have earlier suggested a direct coordination of RuBP to the Cu^{2+} ion (Brändén et al., 1984b). Complex C has an EPR spectrum that is identical with that obtained if D-3-PGA, the product of the RuBP carboxylase/oxygenase reaction, is added to the Cu^{2+}-incubated enzyme in the absence of RuBP. Therefore, it seems reasonable to suggest that complex C has D-3-PGA coordinated to the Cu^{2+} ion. The number of ligands provided by RuBP or D-3-PGA cannot, however, be established by these kinds of experiments.

As shown by the $^{17}O_2$ effect on the EPR spectrum, the intermediate complex B has one oxygen atom, originating from molecular oxygen, coordinated to the Cu^{2+} ion. It is not likely that this oxygen atom comes from the unreacted dioxygen molecule, since protein-bound Cu^{2+} is not expected to have strong dioxygen binding properties (Spiro, 1981). The absence of any effect on the addition of H_2O_2 makes H_2O_2 highly improbable as the compound liganded to Cu^{2+} in complex B. Phosphoglycolate (containing incorporated ^{17}O) and $H_2{}^{17}O$ formed in the reaction between RuBP and $^{17}O_2$ may give rise to the broadening effect seen in Figure 4 if one of these products were coordinated to the Cu^{2+} ion. However, phosphoglycolate and H_2O must be formed simultaneously in the RuBP oxygenase reaction (Miziorko & Lorimer, 1983). Since the formation of phosphoglycolate is kinetically separated from the formation of complex B (see Figure 3), neither phosphoglycolate nor $H_2{}^{17}O$ can be the species giving rise to the

broadening effect seen in Figure 4.

In consideration of the results discussed above, O_2, H_2O, H_2O_2, and phosphoglycolate can all be excluded as the ligand giving rise to the $^{17}O_2$ effect on complex B. An attractive candidate is, however, the 2-peroxy-3-oxopentitol 1,5-bisphosphate suggested to be formed in the RuBP oxygenase reaction (Miziorko & Lorimer, 1983). Such a compound must contain incorporated oxygen, derived from the dioxygen molecule, and its formation must precede the production of phosphoglycolate. All these characteristics are similar to those found for complex B. Furthermore, the chemical structure of such an intermediate must be comparable to that of CABP and RuBP, and therefore an enzyme–Cu^{2+} complex having this compound as a ligand might have an EPR spectrum similar to that of complex A or the enzyme–Cu^{2+}–CABP complex.

We now have evidence supporting a direct coordination of RuBP, D-3-PGA, a hydroperoxide intermediate, and CABP to the Cu^{2+} ion of the Cu^{2+}-incubated RuBP carboxylase/oxygenase. These results are in accordance with the observation that oxygen atom(s) of CABP is (are) directly coordinated to the Mn^{2+} ion in the Mn^{2+}-activated enzyme from *Rhodospirillum rubrum* (Miziorko, 1984).

An effect of D_2O was also observed on the EPR spectra of the three complexes (Figure 5 and Table I). The different line widths of the EPR hyperfine lines obtained in the presence of H_2O and D_2O, respectively, are due to the different magnetic moments of ^1H and ^2H, which interact with the spin of the unpaired electron.

Possible protons available for interaction with Cu^{2+} may originate from (a) H_2O, (b) carboxyl or hydroxyl groups from the enzyme, or (c) RuBP or its products. It seems less likely that protonatable groups of the enzyme are responsible for the effect of D_2O since the pK value for such groups must be 8 or higher in order to contain interacting protons. A hydroxyl group from an enzyme residue may also give the observed effect. However, protein residues containing hydroxyl groups have not been found as ligands to Cu^{2+} in copper-containing or copper-substituted proteins (Spiro, 1981), suggesting that these are not the species interacting with the Cu^{2+} ion in complexes A, B, and C. Protons from water molecules or from hydroxyl groups of RuBP or its products are, on the other hand, possible candidates for the observed effect of D_2O. Water has been shown to be coordinated to the Mn^{2+} ion in the enzyme–Mn^{2+}–CABP complex of *R. rubrum* (Miziorko, 1984), and it has also been suggested that H_2O is coordinated to the metal in the Mg^{2+}-activated enzyme (Nilsson & Brändén, 1983). The effect of D_2O was twice as large on complexes A and C compared to that on complex B. We therefore suggest that complexes A, B, and C have at least one molecule of H_2O coordinated to the Cu^{2+} ion. The effect of D_2O was doubled in complexes A and C, which implies that an additional H_2O molecule or hydroxyl group is interacting with the Cu^{2+} ion.

Phosphoglycolate production was measured by the use of high-pressure liquid chromatography. From Figure 6 it is obvious that the rate of phosphoglycolate formation is very low in the presence of the Cu^{2+}-incubated enzyme. However, a RuBP oxygenase reaction is carried out since phosphoglycolate and 3-PGA are formed in a reaction between RuBP and O_2. The three complexes A, B, and C formed in the presence of RuBP are also in accordance with the proposed mechanism for the enzyme (Miziorko & Lorimer, 1983). Complex A, which is observed at high concentrations of RuBP, may have RuBP coordinated to the Cu^{2+} ion. Molecular oxygen is suggested to react with the enediol form of RuBP, yielding a hydroperoxide intermediate. Complex B has some of the characteristics of such an intermediate coordinated to the Cu^{2+} ion. Complex C, obtained at the end of the reaction, has most certainly D-3-PGA, the product of the RuBP oxygenase reaction, coordinated to the Cu^{2+} ion.

Much work is required before the true nature of all three complexes is determined. These complexes must also be compared to complexes formed in the presence of other activating metal ions before it can be acertained that they are generally applicable to the mechanism of RuBP carboxylase/oxygenase.

Acknowledgments

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Registry No. RuBP, 24218-00-6; CABP, 29843-30-9; D-3-PGA, 3443-58-1; oxygen, 7782-44-7.

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