Identification of Ligands to the Metal Ion in Copper(II)-Activated Ribulose-1,5-bisphosphate Carboxylase/Oxygenase by the Use of Electron Paramagnetic Resonance Spectroscopy and ^{17}O-Labeled Ligands†

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Received January 3, 1985

ABSTRACT: Ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase activated with Cu^{2+} forms complexes with 3-phosphoglyceric acid (3-PGA), the transition-state analogue carboxyarabinitol bisphosphate (CABP), and RuBP. By the use of electron paramagnetic resonance (EPR), ligand atoms to Cu^{2+} have been identified in these complexes from the observation of superhyperfine coupling between Cu^{2+} and the nucleus of ^{17}O atoms in labeled compounds. The enzyme–Cu^{2+}–3-PGA complex contains two molecules of 3-PGA. One of these is coordinated to Cu^{2+} with both the hydroxyl group and the carboxyl group while the other 3-PGA molecule is not coordinated to the metal. In the enzyme–Cu^{2+}–CABP complex the carboxyl group in CABP and one water molecule are coordinated to Cu^{2+}. Two of the complexes formed when RuBP is added to the Cu^{2+}-activated enzyme were investigated. One of these is suggested to contain a peroxy intermediate [Brändén, R., Nilsson, T., & Styring, S. (1984) *Biochemistry 23*, 4378]. Here an observed ^{17}O splitting probably arises from an interaction between the C2-hydroxyl group of the intermediate and Cu^{2+}. The other complex contains bound RuBP, and we observed an interaction between Cu^{2+} and either [^{17}O]water or [2-^{17}O]RuBP. EPR parameters of complexes formed between the Cu^{2+}-activated enzyme and various phosphorylated compounds are also presented. These data indicate that the effector molecules are coordinated to the metal with two oxygen atoms.

Ribulose-1,5-bisphosphate (RuBP)[1] carboxylase/oxygenase (EC 4.1.1.39) catalyzes the carboxylation (eq 1) or oxygenation (eq 2) of RuBP:

$$\text{RuBP} + CO_2 + H_2O \rightarrow 2(\text{3-PGA}) + 2H^+ \quad (1)$$

$$\text{RuBP} + O_2 \rightarrow \text{3-PGA} + \text{phosphoglycolate} + 2H^+ \quad (2)$$

These two reactions are the initial enzymatic steps in photosynthetic carbon assimilation and photorespiration, respectively (Lorimer, 1981).

The activated enzyme contains an activator CO_2 ($^ACO_2$) which is bound as a carbamate to a lysine residue and is stabilized by a divalent metal ion (Miziorko, 1979; Lorimer & Miziorko, 1980). Mg^{2+} is the normal activator (Lorimer, 1981), but also Mn^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+} (Christeller, 1981), and Ca^{2+} (Parry et al., 1983) can support activity with varying efficiency. Recently also Cu^{2+} was shown to bind in the metal site and to maintain low carboxylase and oxygenase activities (Brändén et al., 1984a,b).

Strong evidence for direct participation of the metal in catalysis comes from ^{13}C NMR data which indicate that the metal (Mn^{2+}) and the substrate CO_2 are closely situated (Miziorko & Mildvan, 1974). The much lower K_i for O_2 in the Mn^{2+}–enzyme as compared to the Mg^{2+}–enzyme (Christeller, 1981) as well as the F^- inhibition of the enzyme (Nilsson & Brändén, 1984) also supports metal involvement in catalysis. Valuable information about the coordination and function of the metal ion was recently obtained with EPR spectroscopy. Spectroscopic evidence for direct coordination between the metal ion and substrates has been obtained for Cu^{2+}- and Co^{2+}-activated enzyme from spinach (Brändén et al., 1984a,b; Nilsson et al., 1984) and Mn^{2+}-activated enzyme from *Rhodospirillum rubrum* (Miziorko & Sealy, 1980, 1984).

Although absolute requirement for the metal ion was established several years ago, little is known about its binding site and functional role. As the $^ACO_2$–carbamate is stabilized by M^{2+}, direct coordination between them has frequently been implied (Lorimer & Miziorko, 1980; Lorimer, 1981). However, no chemical evidence exists for this, and experiments on the *R. rubrum* enzyme–$^ACO_2$–Mn^{2+}–CABP complex contradicting this hypothesis were reported recently (Miziorko & Sealy (1984).

A nitrogen atom from the enzyme is proposed to coordinate to the metal ion in the H_2O_2-oxidized enzyme–Co^{2+}–CABP complex (Miziorko et al., 1982). A nitrogen ligand, probably a histidine residue (Saluja & McFadden, 1980; T. Nilsson, unpublished results), is also observed in the Cu^{2+}-activated enzyme but is displaced upon the addition of RuBP, PGA, or CABP (Brändén et al., 1984a,b).

Miziorko & Sealy (1984), using ^{17}O-labeled CABP, could demonstrate that in the *R. rubrum* enzyme–Mn^{2+}–CABP complex CABP was coordinated to Mn^{2+} via the C2-hydroxyl group and the carboxyl group. With [^{17}O]water they could also show the existence of water ligand(s) in the same complex.

† This work has been supported by grants from Statens Naturvetenskapliga Forskningsråd. S.S. was supported by a grant from Stiftelsen Bengt Lundquists Minne. We are also grateful for a grant from Magnus Bergwalls stiftelse that allowed the ^{17}O investigations.

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[1] Abbreviations: EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; RuBP, ribulose 1,5-bisphosphate; CABP, 2-carboxyarabinitol 1,5-bisphosphate; CRBP, 2-carboxyribitol 1,5-bisphosphate; DHAP, dihydroxyacetone phosphate; 2-PGA, 2-phosphoglyceric acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; *R. rubrum*, *Rhodospirillum rubrum*; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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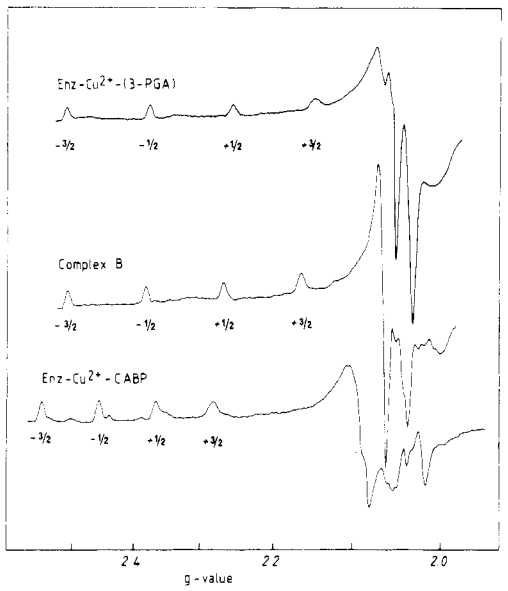


FIGURE 1: EPR spectra of enzyme–Cu^{2+} complexes prepared with 3-PGA, CABP, and RuBP. Complex B is obtained with RuBP and is suggested to be the enzyme–Cu^{2+}–2-peroxy-3-oxopentitol bisphosphate complex. The complexes were prepared as described under Materials and Methods. The spectra were recorded with a modulation amplitude of 0.8 mT and a microwave power of 10 mW at 77 K. The M_I values for the hyperfine lines are indicated.

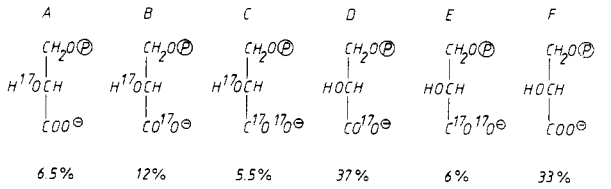
The addition of RuBP to Co^{2+}-activated enzyme from spinach resulted in changes in the Co^{2+} coordination (Nilsson et al., 1984). Two enzyme forms with distorted metal coordination were observed during turnover, suggesting that the carboxylase reaction proceeds via inner-sphere substrate or intermediate complexes. This proposal was further strengthened by the observation of an anisotropic spectrum from the enzyme–Co^{2+}–CABP complex.

The peptide around lysine-201 (which forms the activator–carbamate in the spinach enzyme) contains several carboxylic side chains which have been suggested to bind to the metal ion (Lorimer, 1981). Oxygen ligands from the enzyme (most probably carboxylates) were recently suggested in spinach enzyme–Cu^{2+} complexes with RuBP, CABP, or 3-PGA (Brändén et al., 1984a,b). The EPR spectra of those complexes (Figure 1) all have unusually narrow low-field hyperfine lines (0.8–1.0 mT) due to coordination of oxygen atoms only. The narrow hyperfine lines enabled us to directly observe superhyperfine coupling from ^{17}O to Cu^{2+} in one complex (Brändén et al., 1984b). The coupling was suggested to be derived from coordination of the peroxy group in [2-^{17}O,^{17}O]peroxy-3-oxopentitol bisphosphate to Cu^{2+}. This compound, in which the peroxy group is derived from O_2, is a postulated intermediate in the oxygenase reaction (Lorimer et al., 1973).

The successful ^{17}O investigation of the *R. rubrum* enzyme–Mn^{2+}–CABP complex (Miziorko & Sealy, 1984) prompted us to perform similar experiments in the spinach Cu^{2+}–enzyme since superhyperfine coupling from ^{17}O ligands is expected to be better resolved in the Cu^{2+}–enzyme than in the Mn^{2+}–enzyme.

In this paper we report experiments in which ^{17}O-labeled RuBP, CABP, 3-PGA, and H_2O have been used to further characterize the EPR-distinguishable complexes of the Cu^{2+}–enzyme. Several direct coordinations between those substances and Cu^{2+} in the active site are demonstrated. Results obtained with different effector molecules are also included that allow us to draw conclusions about the flexibility in the active site around the metal ion.

Chart I



Materials and Methods

Materials. RuBP carboxylase/oxygenase was purified from spinach as described earlier (Nilsson & Brändén, 1984). The protein concentration was calculated from the absorption at 280 nm and $A_{280}(1\%) = 16.4$ (Paulsen & Lane, 1966). Phosphorylated effectors, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid (HEPPS), GAPDH, triosephosphate isomerase, and α-glycerophosphate dehydrogenase were obtained from Sigma Chemical Co. Dihydroxyacetone phosphate was found to contain less than 0.1% glyceraldehyde 3-phosphate when checked with GAPDH. Phosphoglycerate kinase was prepared in this department by Dr. L. Arvidsson. CABP was prepared as described in Pierce et al. (1980). CRBP was a kind gift from Dr. G. Lorimer, Du Pont. ^{63}CuO was obtained from Oak Ridge Laboratory and [^{17}O]water from Merck Sharp & Dohme (enrichment according to the manufacturer 50.7%). The enrichment of ^{17}O in the water, before dilution, was estimated to be 51% (±2%) from the EPR spectrum of the enzyme–Cu^{2+}–CABP complex prepared in [^{17}O]water (see Results and Figure 3). All other chemicals were of analytical grade.

Preparation of ^{17}O-Labeled Compounds. The carboxyl group in CABP was labeled with ^{17}O from water by two cycles of delactonizing and lactonizing purified CABP in $H_2{}^{17}O$ (Miziorko & Sealy, 1984). The final ^{17}O label in the carboxyl group was approximately 25% as calculated from the dilutions with unlabeled water during the preparation procedure.

RuBP carboxylase was used to prepare 3-PGA labeled with ^{17}O in the carboxyl group and in the hydroxyl group. RuBP (4 mM), 50 mM HEPPS–NaOH buffer (pH 8.0), and 25 mM HCO_3^- were dissolved in [^{17}O]water in the presence of carbonic anhydrase to ensure complete equilibration of HCO_3^- with [^{17}O]water. Activated RuBP carboxylase and Mg^{2+} were added, and the enzymatic reaction was allowed to proceed to completion. In this experiment the water finally contained 48% ^{17}O. Five kinds of differently labeled 3-PGA molecules exist due to the reaction mechanism of RuBP carboxylase [see, for example, Lorimer (1981, p 368) for isotopic labeling patterns]. In Chart I we show these isotopically distinct forms and the percentage of each [assuming complete equilibration between [2-^{17}O]RuBP and CO_2 with [^{17}O]water (48% enrichment)]. In addition to the labeled 3-PGA molecules 33% of the molecules become unlabeled.

Exclusive ^{17}O label in the hydroxyl group of 3-PGA was achieved with the use of glycolytic enzymes. DHAP (0.75 mg) was dissolved in 20 μL of [^{17}O]water and allowed to equilibrate overnight. This procedure resulted in 48% label in the keto group at C-2 in DHAP. DHAP was thereafter converted to 3-PGA with a coupled enzymatic system containing 10 mM Na_2HAsO_4, 4 mM NAD^+, triosephosphate isomerase, and GAPDH in 2 mL of 50 mM Tris-HCl (pH 7.7) (Herlihy et al., 1976). The reaction was performed at 35 °C and was started by the addition of 20 μL of DHAP in [^{17}O]water. After 20 s the reaction was quenched with 0.2 mL of 5 M $HClO_4$. $KClO_4$ was precipitated with the addition of 0.2 mL of 5 M KOH and was removed, together with denatured protein, by centrifugation. The sample was adsorbed on a

Dowex AG 1-X2 (1 × 20 cm) column. The column was eluted with 10 mL of distilled water and thereafter 150 mM HCl in 50 mM HCl overnight (Brändén et al., 1980). The fractions containing 3-PGA were identified with an enzymatic assay (Racker, 1963) and pooled. 3-PGA was precipitated in ethanol as the Ba salt (Pierce et al., 1980). Approximately 10% of the DHAP was recovered as 3-PGA with this procedure.

^{17}O in the keto group of DHAP exchanges rapidly when DHAP is transferred to the reaction mixture. From the half-times for the hydration and dehydration reactions (Reynolds et al., 1971) we calculated the resulting ^{17}O label in the hydroxyl group of 3-PGA to between 13 and 20%. Less than 0.5% of the carboxylic oxygens are labeled with this procedure.

[2-^{17}O]RuBP was prepared by dissolving solid RuBP in [^{17}O]water. The solution was incubated at room temperature for at least 30 min before use to ensure complete equilibration of the keto oxygen atom with the medium.

Preparation of Enzyme–Cu^{2+} Complexes. The enzyme–Cu^{2+} complexes were prepared essentially as described earlier (Brändén et al., 1984a,b). Enzymes (200–400 μM protomers) were incubated with 25 mM $KHCO_3$ for 10 min in 50 mM HEPPS–NaOH (pH 8.0). Thereafter, $^{63}CuCl_2$ was added to a final concentration reaching 70–80% of the protomer concentration. Two minutes after the addition of Cu^{2+}, RuBP, 3-PGA, CABP, or effector molecules were added. The samples were frozen in EPR tubes at 77 K 2–5 min afterward (CABP 60 min).

In experiments where the coordination of water to Cu^{2+} was investigated the enzyme was concentrated to 1.7–2.4 mM protomers in a collodion bag (Sartorious). The enzyme was thereafter diluted 5–10 times in buffer prepared from [^{17}O]-water in order to maintain high enrichment in the water. The Cu^{2+} complexes were prepared as described above. This procedure resulted in approximately 40% ^{17}O enrichment in the water and a concentration of about 200 μM enzyme protomers.

3-PGA Analysis. High-pressure liquid chromatography (HPLC) was used for determination of 3-PGA concentration as described earlier (Brändén et al., 1984a). 3-PGA was also determined with an enzymatic assay in which 3-PGA was consumed by the coupled action of phosphoglycerate kinase, GAPDH, triosephosphate isomerase, and α-glycerophosphate dehydrogenase in an assay medium containing 4 mM ATP, 4 mM Mg^{2+}, 0.25 mM NADH, and 0.1 mM DTT in 50 mM Tris-HCl (pH 7.6). The concentration of 3-PGA was determined from the consumption of NADH which was measured at 340 nm.

EPR Measurements. EPR spectra at 77 K and 9.4 GHz were recorded with a Bruker ER 200D-SRC spectrometer equipped with an Aspect 2000 computer. EPR spectra at 31 K and 9.1 GHz were recorded with a Varian E-9 spectrometer interfaced to a Nova 3 minicomputer (Data General) for signal averaging from repeated scans. Care was taken not to overmodulate the narrow lines. The spectra in Figures 2–4 are all recorded with a modulation amplitude of 0.63 mT and a microwave effect of 5 mW at 31 K.

EPR Simulations. EPR spectra were simulated assuming Gaussian line shape. The line widths at half-amplitude of the superhyperfine lines, due to the interaction between the nucleus of ^{17}O and Cu^{2+}, were assumed to be equal to that of the hyperfine line in the absence of ^{17}O.

For comparison of simulated and experimental spectra the amplitudes of the "head peaks" were normalized to the same value. The amplitudes of the ^{17}O satellites were then compared, and the deviations are expressed as percentages.

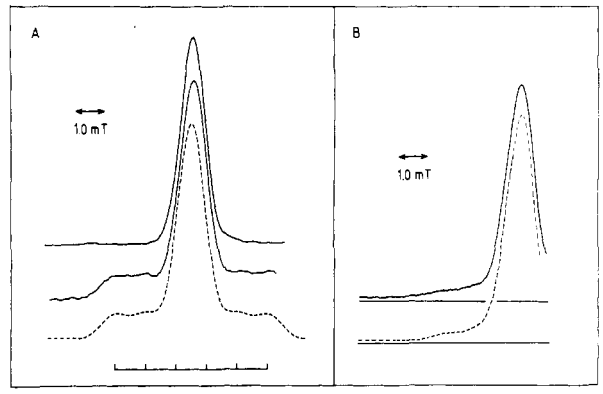


FIGURE 2: EPR spectra of the enzyme–Cu^{2+}–3-PGA complex. (A) Comparison of spectra obtained with unlabeled 3-PGA (upper spectrum) and doubly labeled 3-PGA (lower spectrum). The dotted curve is the simulated spectrum assuming one hydroxyl ligand and one carboxyl ligand originating from the same molecule of 3-PGA. The labeling pattern used in the simulation is described under Results. The superhyperfine splitting pattern is indicated. The hyperfine line at $M_I = -1/2$ is shown. (B) The experimental spectrum obtained with 3-PGA containing 13–20% ^{17}O exclusively in the C2-hydroxyl group and a simulated spectrum (dashed line) using a superhyperfine splitting constant of 1.0 mT and assuming 15% ^{17}O label in one hydroxyl ligand coordinated to Cu^{2+}. The straight line below the experimental spectrum is drawn to help in the comparison with the simulated spectrum. The hyperfine line at $M_I = -3/2$ is shown.

RESULTS

Stoichiometry of 3-PGA in the Enzyme–Cu^{2+}–3-PGA Complex. Cu^{2+}-activated RuBP carboxylase/oxygenase from spinach forms a strong 3-PGA complex with a characteristic Cu^{2+} EPR spectrum (Figure 1) indicative of oxygen ligands only (Brändén et al., 1984b). This complex is stable to gel filtration, thereby facilitating determination of the stoichiometry of 3-PGA in the complex. In three separate experiments the complex was prepared with excess 3-PGA (5 mM). Unbound Cu^{2+} and 3-PGA was removed by gel filtration, and the fractions containing the enzyme–3-PGA complex were immediately frozen in EPR tubes.

The concentration of EPR-detectable Cu^{2+} in the samples was determined by single line integration of the low-field hyperfine lines according to Aasa & Vänngård (1975). The protein was removed by denaturation, and 3-PGA was determined by HPLC or the enzymatic assay. The stoichiometry of 3-PGA to Cu^{2+} in the complex was found to be 1.8, 1.5, and 2.0 in the experiments. We therefore conclude that the enzyme–Cu^{2+}–3-PGA complex contains two molecules of 3-PGA per Cu^{2+}.

Coordination to Cu^{2+} in the Enzyme–Cu^{2+}–3-PGA Complex. With the use of ^{17}O-labeled compounds, ligands to Cu^{2+} can be identified. When a ^{17}O-containing group is directly coordinated to the Cu^{2+} ion, this might result in splitting or broadening of the hyperfine lines in the EPR spectrum. In order to investigate the possible coordination of 3-PGA to Cu^{2+} in the enzyme–Cu^{2+}–3-PGA complex, we prepared 3-PGA labeled with ^{17}O in (i) both the C2-hydroxyl group and the carboxyl group or (ii) the C2-hydroxyl group exclusively.

The syntheses were accomplished with either RuBP carboxylase (i) or glycolytic enzyme (ii) and resulted in labeling as presented under Materials and Methods.

The enzyme–Cu^{2+}–3-PGA complex was prepared with ^{17}O-labeled 3-PGA (i or ii). In Figure 2A one of the hyperfine lines in the EPR spectrum is shown for unlabeled and double labeled (i) PGA complexes. Splitting of the line is observed in the ^{17}O-labeled complex. The splitting can only be derived

Table I: Superhyperfine Coupling Constants for Coupling of ^{17}O to Cu^{2+} in Enzyme–Cu^{2+} Complexes

complex	ligand to Cu^{2+}	superhyperfine coupling constant[a] (mT)
enzyme–Cu^{2+}–3-PGA	the oxygen atom in the C2-hydroxyl group	1.0
	one oxygen atom in the carboxyl group	1.01
enzyme–Cu^{2+}–CABP	one oxygen atom in the carboxyl group	0.82
	one water molecule	1.00
complex A[b]	H_2O or the keto group in RuBP	0.95
complex B[c]	the C2-hydroxyl group or a slowly exchangeable water molecule	1.01
	one oxygen atom in the peroxy group	0.96

[a] The superhyperfine coupling constants are determined from the experimental spectra directly. [b] Complex A contains one molecule of RuBP (Brändén et al., 1984a). [c] Complex B is suggested to contain the intermediate in the oxygenase reaction, 2-peroxy-3-oxopentitol bisphosphate (Brändén et al., 1984b).

from direct coordination of ^{17}O atoms in 3-PGA to Cu^{2+}. Since ^{17}O has a nuclear spin, $I = {}^5/_2$, six superhyperfine lines are expected. Four of these are partially resolved, and their positions are indicated in the spectrum. The observed superhyperfine coupling constant is 1.01 mT (Table I).

In the experiment presented in Figure 2A the 3-PGA used was doubly labeled. Therefore, different ^{17}O-splitting patterns are expected, dependent on the number and nature of ^{17}O-labeled ligands that coordinate to Cu^{2+}. Since the enzyme–Cu^{2+}–3-PGA complex contains two molecules of 3-PGA, each doubly labeled, many combinations can exist with one, two, three, or even four different ^{17}O ligands coordinated to the Cu^{2+} ion. The expected ^{17}O-splitting patterns for the different ligand situations were determined. Care must be taken in those calculations, since RuBP carboxylase was used to produce labeled 3-PGA from [^{17}O]RuBP, $^{17}CO_2$, and [^{17}O]water. Five differently labeled forms of 3-PGA (besides unlabeled 3-PGA) were produced (Chart I) which must be treated individually since they give rise to different splitting patterns. The theoretically calculated splitting patterns, the experimentally determined line width, and the superhyperfine coupling constant from Figure 2A were used to simulate the following ligand combinations: (a) one ligand from the C2-hydroxylic group of 3-PGA, (b) one ligand from the carboxyl group of 3-PGA, (c) two ligands with similar couplings from the C2-hydroxylic groups of the two 3-PGA molecules, (d) two ligands with similar couplings from the two different carboxylic groups, (e) one C2-hydroxylic and one carboxylic ligand with similar couplings and originating from the same 3-PGA molecule, (f) one C2-hydroxylic and one carboxylic ligand with similar couplings but originating from different 3-PGA molecules, and (g) cases with sets of three or four ligands.

From Chart I it is seen that, when only the C2-hydroxyl group is coordinated to the metal ion (a), 24% of the bound 3-PGA molecules will give rise to splitting in the EPR spectrum. The simulated spectrum in this case (not shown) deviates 45% (see Materials and Methods) from the experimental spectrum since the amplitudes of the simulated superhyperfine lines are too small. The splitting pattern expected from one carboxylic oxygen coordinated to Cu^{2+} (b) is more complicated to calculate. Forms C and E (Chart I) will contribute to the splitting in all cases since both of the carboxylic oxygens are labeled. However, forms B and D will contribute to the splitting in half of the cases since only one carboxylic oxygen is labeled. Thus, we will expect splitting from 36% of the 3-PGA molecules [from Chart I: 6%(B/2) + 5.5%(C) + 18.5%(D/2) + 6%(E)]. The amplitude of the ^{17}O satellites in the simulated spectrum (not shown) is too small to account for the experimental spectrum (the deviation is 25%). With this procedure we calculated the simulated spectra for (a)–(g). The simulated spectrum for e gave a very close fit (the deviation is 3%) to the experimental spectrum (Figure 2A). In this case we expect 11.5% of the complexes to contain two ^{17}O atoms coupled to Cu^{2+} [6%(B/2) + 5.5%(C)] and 37% to contain one ^{17}O atom coordinated to the Cu^{2+} ion [6.5%(A) + 6%(B/2) + 18.5%(D/2) + 6%(E)]. In (f) 8.6% of the complexes are expected to have two ^{17}O atoms coordinated to Cu^{2+} and 43% to contain one ^{17}O ligand. The simulated spectrum for this case deviates 25% from the experimental spectrum. In all cases in (g) the divergence was greater than 45%.

The deviations are determined within ±10% (including base-line error). Therefore, the observed deviations in cases a–d, f, and g are significantly too large to account for the experimental spectrum. From this we consider case e to reflect the situation in the 3-PGA complex.

The 3-PGA complex was prepared also with 3-PGA labeled in the hydroxyl group exclusively (ii). Here the precise amount of labeling is unimportant since the mere existence of a ^{17}O splitting give exclusive evidence for a direct interaction between Cu^{2+} and the hydroxyl group in 3-PGA. In Figure 2B one hyperfine line from the enzyme Cu^{2+}–[2-^{17}O]-3-PGA complex is compared to the same line from an unlabeled complex. In this experiment the C2-hydroxyl group contained 13–20% ^{17}O label. The shoulder on the low-field side of the peak originating from ^{17}O splitting is clearly observable although the superhyperfine couplings are not completely resolved. The experimental spectrum is well simulated with the assumption that the splitting is due to the coordination of one C2-hydroxyl group (which is labeled with 15% ^{17}O) to Cu^{2+}.

It was of interest also to investigate the possible coordination of the activator CO_2 or water molecules to Cu^{2+} in the enzyme–Cu^{2+}–3-PGA complex. This was therefore prepared in a medium containing 20 mM HCO_3^- and 50 mM HEPPS–NaOH (pH 8.0) in [^{17}O]water. Complete equilibration of HCO_3^- with the [^{17}O]water was assured by the addition of carbonic anhydrase 30 min before RuBP carboxylase was added. The PGA complex was prepared with unlabeled 3-PGA. Therefore, any ^{17}O splitting in the EPR spectrum would be derived from either $^{A}C^{17}O_2$ or [^{17}O]water ligands. However, no splitting or broadening could be observed although the spectrum was obtained with low modulation to ensure observation of possible smaller superhyperfine couplings. The most straightforward interpretation is that neither $^{A}CO_2$ nor H_2O coordinate to Cu^{2+} in the 3-PGA complex. In this complex one of the 3-PGA molecules contributes with two ligands, one C2-hydroxyl group, and one carboxylic oxygen, while the other ligands are from the enzyme.

Coordination to Cu^{2+} in the Enzyme–Cu^{2+}–CABP Complex. The tight enzyme–Cu^{2+}–CABP complex has an EPR spectrum characterized by narrow hyperfine lines and a high-field part indicative of separated g_x and g_y values. Together with an unusually high g_z value ($g_z = 2.41$) and a small hyperfine splitting constant ($A_z = 10.3$ mT) these parameters indicate that Cu^{2+} has only oxygen ligand atoms in an arrangement that deviates from tetragonal symmetry.

[*carboxy*-^{17}O]CABP was used to prepare the enzyme–Cu^{2+}–CABP complex. One hyperfine line in the resulting EPR

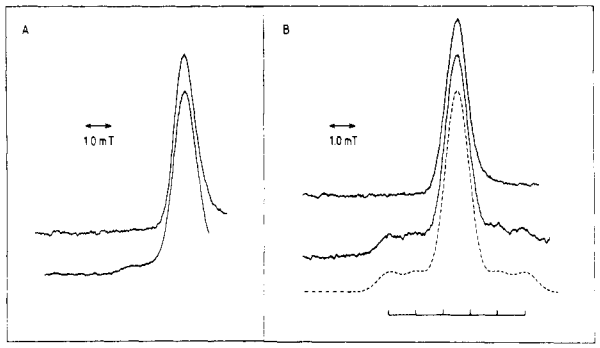


FIGURE 3: EPR spectra of the enzyme–Cu^{2+}–CABP complex. The hyperfine line at $M_I = -3/2$ is shown. (A) Comparison of spectra obtained with unlabeled CABP (upper spectrum) and CABP labeled with 25% ^{17}O in the carboxylic group (lower spectrum). (B) The spectrum obtained from the CABP complex prepared in [^{17}O]water (lower spectrum) (^{17}O enrichment 39.1%) and in unlabeled water (upper spectrum). The dashed curve is a simulated spectrum assuming one ^{17}O ligand and 39.1% ^{17}O label in the ligand. The superhyperfine couplings are partly resolved and are indicated in the figure.

spectrum is presented in Figure 3A. The hyperfine line is distinctly split by the ^{17}O label in CABP. As CABP here is only labeled in the carboxyl group, the effect of ^{17}O on the hyperfine line proves this group to be coordinated to Cu^{2+}. Exact knowledge of the enrichment in this case is therefore not critical. However, with an enrichment of 25% ^{17}O atoms (see Materials and Methods), a superhyperfine splitting constant determined to 0.8 mT, and assuming coordination of one carboxylic oxygen atom, the experimental spectrum is well simulated. We therefore conclude that the carboxyl group in CABP is coordinated to Cu^{2+} in the spinach enzyme–Cu^{2+} complex.

Miziorko & Sealy (1984) presented evidence for a water ligand in the *R. rubrum* enzyme–Mn^{2+}–CABP complex. We prepared a similar spinach enzyme–Cu^{2+}–CABP complex in [^{17}O]water (39.1% ^{17}O). One hyperfine line is shown in Figure 3B. Splitting from ^{17}O is easily observed. In this experiment four superhyperfine lines are resolved, and the coupling constant is 1.0 mT. The experimental spectrum is well simulated assuming 39.1% ^{17}O label and one ^{17}O ligand (Figure 3B) (in this case, the simulated spectrum deviates less than 4% from the experimental spectrum). This indicates that one water molecule is present in the inner sphere of Cu^{2+} in this complex. The data for ^{17}O couplings in the CABP-complexes are collected in Table I.

Coordination to Cu^{2+} in Complexes Formed with RuBP. We have previously reported the formation of two EPR-detectable complexes after the addition of RuBP to Cu^{2+}-activated enzyme (Brändén et al., 1984a,b). Complex A is a stable, inert complex containing one molecule of RuBP per Cu^{2+}. Complex B is a transient, oxygen-dependent enzyme–Cu^{2+} complex, whose decomposition is correlated to the production of phosphoglycolate and 3-PGA. We have proposed complex B to contain the postulated intermediate in the oxygenase reaction 2-peroxy-3-oxopentitol 1,5-bisphosphate.

We prepared complex B from [2-^{17}O]RuBP in [^{17}O]water under oxygen-saturating conditions. Essentially no complex A was formed in this experiment. The EPR spectrum of one hyperfine line from complex B is presented in Figure 4A. The line is split by one ^{17}O ligand as judged from the simulated spectrum (Figure 4A). The nature of this ^{17}O-containing ligand is not obvious. When RuBP is transferred to [^{16}O]-water, the time required for exchange of the ^{17}O label in [2-^{17}O]RuBP is much shorter (Lorimer et al., 1984) than the time needed to produce spectroscopically useful concentrations

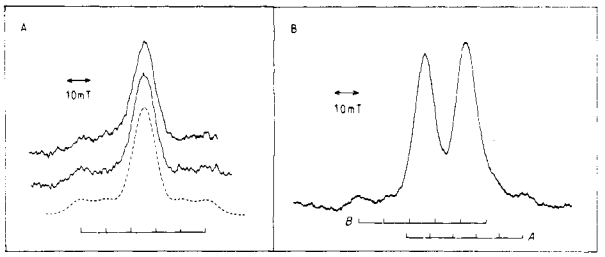


FIGURE 4: (A) EPR spectra of complex B. The lower spectrum was obtained for a sample prepared in [^{17}O]water (47.7% ^{17}O) under oxygen-saturating conditions. The complex was diluted twice in buffer, and the upper spectrum was recorded. The dashed curve is the simulated spectrum obtained by using 47.7% ^{17}O enrichment and assuming one ^{17}O-containing ligand in this complex. The hyperfine line at $M_I = -1/2$ is shown. (B) EPR spectrum of complex A (right peak) and complex B (left peak). The complexes were prepared in 40.3% enriched [^{17}O]water from [2-^{17}O]RuBP. An attempt to identify the superhyperfine couplings in the two complexes is shown. The hyperfine line at $M_I = -1/2$ is shown.

of complex B (about 5 min). Therefore, the experiment was performed in [^{17}O]water. In this case two ^{17}O-labeled species exist in the medium, [2-^{17}O]RuBP, and [^{17}O]water. Under those circumstances the peroxy intermediate in complex B will become ^{17}O labeled in the hydroxyl group at C2. In an attempt to distinguish between the possible ^{17}O ligands, the sample was diluted with unlabeled buffer and allowed to equilibrate 2–8 min before being refrozen. 2(OH)-^{17}O in the peroxy intermediate cannot exchange with the solvent while a water ligand might do so. The splitting remained unaffected by the dilution (Figure 4A) which leads us to suggest that, in complex B, the C2-hydroxyl group in the peroxy intermediate is coordinated to Cu^{2+} [together with the peroxy group as shown earlier (Brändén et al., 1984b) although a slowly exchanging water ligand cannot be excluded].

Also complex A was prepared from [2-^{17}O]RuBP in [^{17}O]water. In this experiment the EPR spectrum is a mixture of complex A and B (Figure 4B), complicating the interpretation. The superhyperfine coupling of ^{17}O to Cu^{2+} in complex B is known from Figure 4A which makes it possible to assign ^{17}O splitting in complex A due to one ^{17}O ligand. An attempt to identify the superhyperfine couplings is presented in Figure 4B. The sample was diluted twice in unlabeled buffer (data not shown), and within less than 2 min the ^{17}O effect in complex A was reduced by 50% while the ^{17}O effect in complex B remained unaffected as before. Thus, the ^{17}O ligand in complex A equilibrates easily with the bulk water. Also in this case two kinds of labeled molecules exist in the medium, [2-^{17}O]RuBP, and [O^{17}]water. The keto group of RuBP is a candidate for coordination to Cu^{2+}. However, the Mg^{2+}–enzyme does not catalyze wash out of ^{18}O from [2-^{18}O]RuBP via the *gem*-diol form. Instead, the addition and elimination of water at C2 of RuBP is stereochemically directed in the active site (Lorimer et al., 1984). Therefore, we do not expect the tightly bound RuBP in complex A (Brändén et al., 1984a) to exchange the label in the keto group with the medium in the dilution experiment if this group was coordinated to the metal. This would exclude the keto group as a Cu^{2+} ligand in complex A. Instead the most likely explanation for our results is that a water molecule, which can exchange readily with the bulk water, is coordinated to Cu^{2+} in complex A.

Enzyme–Cu^{2+}–Effector Complexes. Phosphorylated molecules like fructose 1,6-bisphosphate, 6-phosphogluconate, NADPH, and others are suggested to bind in the active site, thereby stabilizing the activated enzyme (Badger & Lorimer, 1981). Most effector molecules are competitive inhibitors with respect to RuBP, suggesting that they bind to the same site.

Table II: EPR Parameters of Complexes Formed between Cu^{2+}-Activated RuBP Carboxylase/Oxygenase and Various Effector Molecules[a]

compound	$g_\parallel$	$A_\parallel$ (mT)
Group A: Substances That Form Enzyme–Cu^{2+} Complexes[b]		
3-PGA	2.31	14.3
2-PGA	2.32	14.3
phosphohydroxypyruvic acid	2.31	14.4
fructose 1,6-bisphosphate	2.36	11.2
6-phosphogluconate	2.38	12.5
ribose 5-phosphate	2.29, 2.31	15.2, 13.4[c]
CRBP	2.34	12.1
CABP	g_z = 2.41	A_z = 10.3[d]
sedoheptulose 1,7-bisphosphate	g_z = 2.38	A_z = 11.1
	g^y = 2.10	A^y = 2.5
	g_x = 2.03–2.05	A_x[e]
Group B: Substances That Do Not Form Complexes[f]		
glyceraldehyde 3-phosphate	fructose 6-phosphate	
α-glycerophosphate	glucose 6-phosphate	
phosphoenolpyruvate	NADPH	
DHAP	phosphate	
phosphoglycolate	pyrophosphate	

[a] The complexes were prepared as described under Materials and Methods. Both stoichiometric (200–400 μM) and excess (5 mM) amounts of the effector molecules were used in all cases. For compounds in group B both shorter times (2 min) and longer times (1 h) were used for complex formation. [b] Group A: substances that form enzyme–Cu^{2+} complexes with narrow hyperfine lines in the low-field part of the EPR spectrum (line width approximately 1.0 mT in all cases). [c] Two different complexes are formed. [e] Not resolved. [f] Group B: substances that do not form complexes with narrow lines in the EPR spectrum.

Enzyme–Cu^{2+}–effector complexes might then be used to investigate effector binding in the active site.

EPR spectra of enzyme–Cu^{2+} complexes with different effector molecules were recorded. The results are collected in Table II. Here the compounds are organized according to their ability to form EPR spectra with narrow hyperfine lines. Many of the substances form complexes with such spectra. The most reasonable explanation for this is that the effector molecule is coordinated directly to Cu^{2+} with one or two oxygen atoms in analogy to 3-PGA, CABP, and the peroxy intermediate in complex B.

Discussion

EPR spectra of RuBP carboxylase/oxygenase was first obtained by Miziorko & Sealy (1980) for the spinach enzyme–Mn^{2+}–CABP complex. The authors were able to suggest distorted metal coordination although the spectra gave relatively little information about the ligands around the metal. We later expanded the EPR investigations of the spinach enzyme to the Cu^{2+}- and Co^{2+}-activated forms and presented EPR spectra of the enzyme–Cu^{2+}–CABP and enzyme–Co^{2+}–CABP complexes (Brändén et al., 1984b; Nilsson et al., 1984). These spectra are derived from complexes with low symmetry so the original suggestion of distorted metal coordination in the spinach enzyme–Mn^{2+}–CABP complex (Miziorko & Sealy, 1980) is obviously extensible to other activator metals as well.

We investigated the oxygenase reaction in the spinach Cu^{2+}–enzyme (Brändén et al., 1984b) and presented the EPR spectrum of complex B. Direct coordination between Cu^{2+} and the peroxy group in complex B was proven by the observation of ^{17}O-superhyperfine coupling between Cu^{2+} and the suggested intermediate in the oxygenase reaction. The ^{17}O technique was also used by Miziorko & Sealy (1984), who demonstrated direct coordination between Mn^{2+} and CABP in the *R. rubrum* enzyme. In this complex, Mn^{2+} is coordinated to the C2-hydroxyl group and the carboxyl group of CABP. The authors also observed one or more water ligands.

In this paper we present results for the spinach enzyme–Cu^{2+}–CABP complex, which confirm the findings by Miziorko & Sealy (1984). We are able to identify the carboxyl ligand also in the Cu^{2+} complex. We can also demonstrate one water ligand in this case. Miziorko and Sealy were unable to determine the number of water ligands in the *R. rubrum* enzyme–Mn^{2+}–CABP complex due to unfavorable line widths and superhyperfine coupling constants. In the Cu^{2+}–enzyme the hyperfine lines are very narrow, making superhyperfine splitting from [^{17}O]water easily observable (Figure 3B). This allows us to establish one water molecule in the inner sphere of Cu^{2+}. We have not investigated the possible coordination of the C2-hydroxyl group. However, our data are very similar to those of Miziorko and Sealy, leading us to suggest that the C2-hydroxyl group is coordinated to the metal ion also in the Cu^{2+} complex. The identification of the hydroxyl ligand in this position also in the enzyme–Cu^{2+}–3-PGA complex further strengthens this hypothesis.

The available structural information about the ligands around the metal ion in the CABP complex is collected in Figure 5A. The geometry in the complex was suggested to be an arrangement with low symmetry similar to that of the Mn^{2+} complex (Miziorko & Sealy, 1980). The spectral parameters of the enzyme–Cu^{2+}–CABP complex are quite similar to those of the enolase–Cu^{2+}–2-PGA complex (Dickinson et al., 1980). By analogy with a Cu^{2+}-doped Zn^{2+}–Tutton salt (Bleaney et al., 1955), this spectrum was suggested to be derived from a 6-coordinated Cu^{2+} with low symmetry. However, similar parameters may probably be derived also from 5-coordinated complexes of low symmetry as judged from the analysis by Bencini et al. (1979) of Cu^{2+} complexes with geometries intermediate between square planar and trigonal bipyramidal. The superhyperfine coupling from the carboxyl group of CABP to Cu^{2+} (0.8 mT) is smaller than the other observed couplings in the enzyme–Cu^{2+} complexes (around 1.0 mT; Table I) (this work; Brändén et al., 1984b) which might indicate an unusually long distance between the carboxyl group and the Cu^{2+} ion or that the carboxylic ligand is not situated in the equatorial plane. Both situations are equivalent to a rhombic distorsion and might explain the rhombic EPR spectrum of the CABP complex.

No enzymatic ligands have been identified although carboxylates or hydroxylic residues around lysine-201 are likely candidates. In Figure 5A we have indicated two enzyme ligands and left one position empty (if Cu^{2+} is 6-coordinated). The activator carbamate is placed close to the metal ion although the carbamate probably is not directly coordinated to the metal since coupling from ^{17}O-labeled $^{A}CO_2$ could not be detected either in the spinach enzyme–Cu^{2+}–3-PGA complex (this work) or in the *R. rubrum*–Mn^{2+}–CABP complex (Miziorko & Sealy, 1984).

The enzyme–Cu^{2+}–3-PGA complex (Figure 5B) is fairly well characterized. It contains two 3-PGA molecules which probably are bound with their phosphate groups in the phosphate sites for RuBP. This mode of 3-PGA binding was also suggested from a kinetic investigation of 3-PGA inhibition of the carboxylase activity (Laing & Christeller, 1980).

We have identified two Cu^{2+} ligands from 3-PGA. One is a C2-hydroxyl group and the other is a carboxylic oxygen atom. Both ligands originate from the same molecule of 3-PGA. This makes the complex analogous to the CABP complex (compare parts A and B of Figure 5). The EPR spectrum of the enzyme–Cu^{2+}–3-PGA complex is less rhombic than that

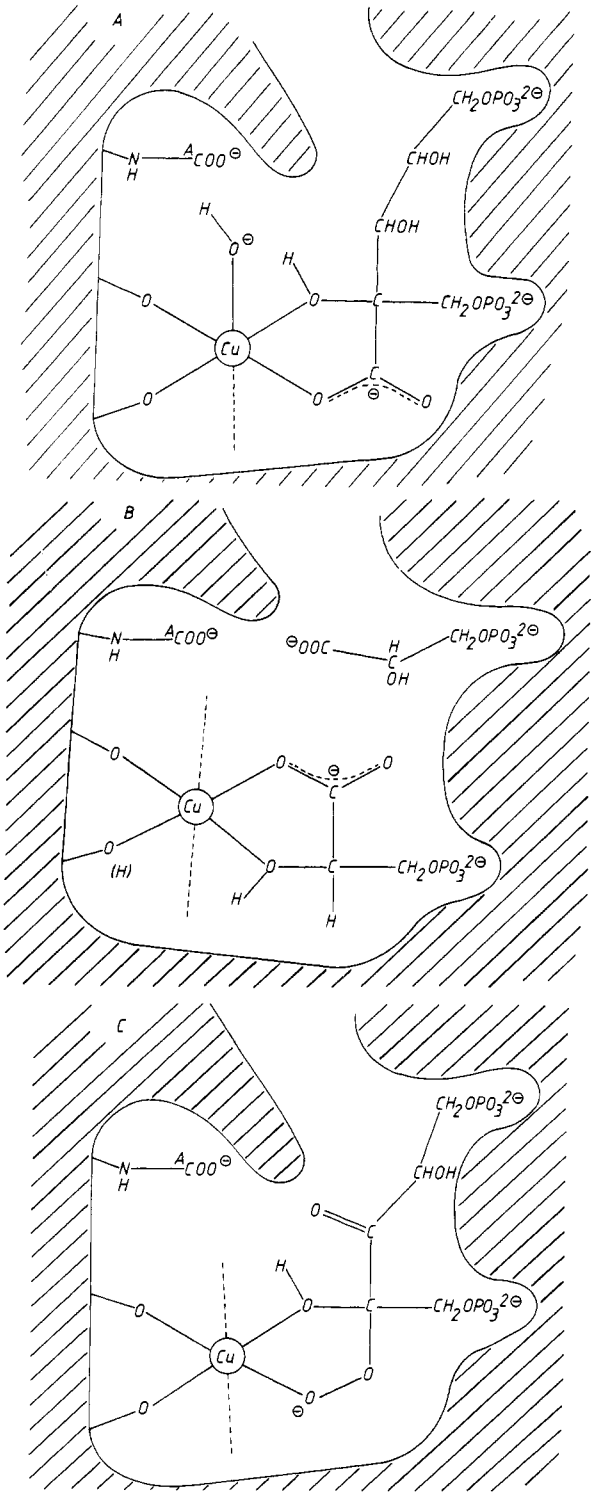


FIGURE 5: Schematic models for the ligand situation around the metal ion in enzyme–Cu^{2+} complexes. (A) Enzyme–Cu^{2+}–CABP complex. (B) Enzyme–Cu^{2+}–3-PGA complex. (C) Complex B, enzyme–Cu^{2+}–2-peroxy-3-oxopentitol bisphosphate complex.

of the CABP complex, indicative of a more symmetric metal center. Obviously CABP, being fixed at several positions by the enzyme, changes the metal coordination. Contrary to the situation in the CABP complex we cannot observe coupling from a water molecule to Cu^{2+} in the 3-PGA complex. However, Cu^{2+} in this complex probably has tetragonal symmetry. In tetragonal complexes any ^{17}O-superhyperfine couplings from axial ligands would be less than 10% of those from equatorial ligands due to the low spin density in the $^3d_{z^2}$ orbital (Denium & Vänngård, 1975; Getz & Silver, 1974). We have therefore left the two axial positions open in our model for the 3-PGA complex (Figure 5B).

The two identified 3-PGA ligands are in the equatorial plane as they otherwise would not be observable. Furthermore, the superhyperfine couplings (about 1.0 mT) are similar to those observed earlier for equatorial ligands to Cu^{2+} (1.2 mT in Cu^{2+}–acetyl acetonate (Denium & Vänngård, 1975), 0.96 and 0.78 mT in a Cu^{2+}-doped zinc Tutton salt (Getz & Silver, 1974) and 1.0 mT in a Cu^{2+} hydroxyquinolinate complex (Lorenz et al., 1972). The two ligands provided by the enzyme as indicated in the model must be in the plane. Otherwise these positions would have been filled from 3-PGA or water giving rise to observable ^{17}O splittings in the experimental spectra. In an earlier paper (Brändén et al., 1984b) we reported narrowing effects on the hyperfine lines in the EPR spectrum when the 3-PGA complex was prepared in D_2O. This effect was suggested to depend on the presence of two exchangeable protons in the vicinity of Cu^{2+}. In ENDOR experiments performed in this department (S. Styring and R. Aasa, unpublished results) we have observed two exchangeable protons in this complex, thus confirming our original hypothesis. One of these protons obviously is the hydroxylic proton from 3-PGA while the other is more difficult to identify. However, as also proton couplings are smaller from axial positions, the most likely origin is one of the enzymatic ligands in the equatorial plane (the proton is depicted in parentheses in Figure 5B).

A model of complex B is presented in Figure 5C, where we have drawn the suggested intermediate in the oxygenase reaction, 2-peroxy-3-oxopentitol bisphosphate. The peroxy group is placed in the same position as the carboxyl group of CABP. In analogy to the situation in the CABP complex we have also placed the C2-hydroxyl group in the coordination sphere of Cu^{2+} although we cannot rule out the presence of a nonexchangeable water ligand instead of this hydroxyl group (see Results).

We also investigated the metal site with use of different phosphorylated effector molecules that bind near the metal ion and therefore might influence the EPR spectra. The most obvious spectral change is the formation of enzyme–Cu^{2+}–effector complexes with narrow hyperfine lines. In such complexes the effector simultaneously binds to Cu^{2+} and replaces the nitrogen ligand in the Cu^{2+}-activated enzyme. The effector in complexes that do not show narrow hyperfine lines in the EPR spectra either lacks oxygen atoms correctly oriented to replace the nitrogen or does not bind close to the metal ion.

The spectral data of the investigated effector complexes are collected in Table II. Of particular interest are the results obtained with phosphorylated three-carbon compounds. Of these, the three carboxylic acids, 3-PGA, 2-PGA, and phosphohydroxypyruvate form complexes with narrow hyperfine lines. The compounds that lack the carboxyl group, DHAP, glyceraldehyde 3-phosphate, and glycerol 1-phosphate, do not form such complexes. These results demonstrate that a carboxyl group is necessary for replacement of the nitrogen ligand, probably by competition for the same ligand position on Cu^{2+}. Phosphoenolpyruvate and phosphoglycolate, although having a carboxyl group, are unable to form complexes with narrow hyperfine lines in the EPR spectrum. However, these compounds lack hydroxyl or keto groups, which are possible Cu^{2+} ligands together with the carboxyl group. This also suggests that effectors that form complexes with narrow hyperfine lines coordinate to Cu^{2+} via two functional groups in analogy to 3-PGA and CABP.

Fructose 1,6-bisphosphate, 6-phosphogluconate, and sedoheptulose 1,7-bisphosphate are activating effectors and com-

petitive inhibitors to RuBP carboxylase (Badger & Lorimer, 1981; Salujah & McFadden, 1980). From the EPR spectra it is clear that they bind to the metal in the RuBP site, forming well-defined complexes. This binding is evidently the molecular basis for the dual behavior of these substances. Similar conclusions were drawn from kinetic data (Badger & Lorimer, 1981), leading the authors to suggest a model for the role of effectors in the activation of RuBP carboxylase. The spectral data on effector binding presented in this paper support their analysis and strengthen their presented model.

Of interest is the EPR spectrum of the sedoheptulose 1,7-bisphosphate complex which is highly rhombic with three measurable g values (Table II). The spectral parameters are very similar to those recorded by Dickinson et al. (1981) for the enolase–Cu^{2+}–2-PGA complex. They are also quite similar to those estimated for the CABP complex.

Plausible reaction mechanisms for the carboxylase and oxygenase reactions have been published in the last years (Lorimer, 1981; Miziorko & Lorimer, 1983). In both reactions the initial step is the abstraction of the C3 proton of RuBP, thereby forming a reactive species of RuBP, the 2–3 enolate anion. The existence of the enolate anion was recently indicated in the carboxylase reaction (Jaworowski et al., 1984; Bhagwat & McFadden, 1983). The enolate is attacked by CO_2, forming a six-carbon intermediate (Schloss & Lorimer, 1982), or O_2, forming the suggested peroxy intermediate (Lorimer et al., 1973). The intermediates are thereafter cleaved by water or hydroxyl ion attack on C3, ultimately leading to formation of the products.

Our data obtained for the enzyme–Cu^{2+}–effector complexes indicate that the metal environment is flexible. Two metal ligands of varying nature are provided by the effector molecule. The metal is also able to change symmetry from axial in some complexes to highly rhombic in others. This ability to change the metal environment may be important for the catalytic function. As discussed by several authors (Pierce et al., 1980; Nilsson et al., 1984) the metal can participate in several steps in catalysis. The coordinations determined in this paper are important in such considerations.

(1) The C2-hydroxyl group which is shown to coordinate to the metal ion is introduced to the active enzyme as the keto oxygen in RuBP. We propose by analogy that this oxygen atom also in RuBP is directly coordinated to the metal in the active enzyme. In this case the enolate ion formed after extraction of the C3 proton is stabilized by the metal ion which functions as an electron sink. Metal coordination of this oxygen atom also explains its nonexchangeability (exchange would otherwise be expected via the *gem*-diol form) with the solvent (Sue & Knowles, 1978, Lorimer, 1984). It is important to observe that the keto group in RuBP probably is not coordinated to the Cu^{2+} ion in complex A (see Results). This might be the structural basis for the inertness of this complex (Brändén et al., 1984a).

(2) The structure of CABP is analogous to the structure of the six-carbon intermediate (Schloss & Lorimer, 1982). The carboxyl group of CABP is equivalent to the carboxyl group in the intermediate. This carboxyl group originates from the substrate CO_2. If the six-carbon intermediate is coordinated similarly to CABP, metal coordination of the carboxyl group might help in obtaining the correct orientation of the intermediate in the active site. The intermediate is subsequently cleaved by a hydroxyl ion or a water molecule. Nilsson & Brändén (1983) suggested, from kinetic studies of the fluoride inhibition, that the attacking species in hydrolysis is delivered by the metal ion. Since the CABP complex contains a metal-coordinated water molecule, the interesting possibility exists that the metal ion not only correctly aligns the six-carbon intermediate but also delivers the species necessary for its cleavage.

(3) For the oxygenase reaction similar suggestions are plausible. In this case an oxygen molecule attacks the enolate ion of RuBP. The intermediate coordinates to the metal via two ligands and is probably oriented (Figure 5C) similarly to the six-carbon intermediate. The bond between carbon atoms 2 and 3 is thereafter hydrolyzed also in this complex. The origin of the attacking species is not clear. Complex B does not contain a tightly coupled water molecule as was the situation in the CABP complex. However, the presence of an axial water ligand that could be delivered in hydrolysis is an attractive suggestion and not at all unlikely.

(4) The situation in the active site after the cleavage of the six-carbon intermediate before release of the products may resemble the situation in our model for the 3-PGA complex (Figure 5B). The suggested products formed immediately after hydrolysis are 3-PGA (from carbon atoms 3, 4, and 5 of RuBP) and the C2 carbanion of 3-PGA (from CO_2 and carbon atoms 1 and 2 of RuBP) (Pierce et al., 1981; Lorimer, 1981). From the stereochemistry of the carboxylase reaction Pierce et al. (1981) suggested that the configuration of the C2 carbanion is inverted before the carbanion can be protonated to 3-PGA. In the Cu^{2+} complex this 3-PGA molecule is coordinated to the metal via the carboxyl group and the hydroxyl group. If also the carbanion is coordinated in this manner, this would facilitate its formation and catalysis.

The first step in catalysis involves the binding of RuBP. So far we have only discussed coordination of the keto group of RuBP to the metal. However, in many complexes the metal offers two ligand positions to the substrate. If also RuBP is coordinated to the metal in this manner, the C3-hydroxyl group is attractive as a second ligand. This group is close enough to suggest binding. Furthermore, coordination of the oxygens at both carbon 2 and carbon 3 would stabilize the enolate ion of RuBP and direct the electrophilic attack of CO_2 and O_2 to carbon 2 which would be most important for catalysis.

The results presented here show that EPR studies of RuBP carboxylase/oxygenase can provide valuable information about the environment and catalytic function of the metal that otherwise would be difficult to obtain. The question whether results obtained with enzyme activated with Cu^{2+} are relevant also for other activator metals might well arise. However, the results obtained so far with the Cu^{2+}–enzyme (Brändén et al., 1984a,b; this paper) are in full agreement with the EPR results from the Mn^{2+}–enzyme (Miziorko & Sealy, 1980, 1984) and the Co^{2+}–enzyme (Nilsson et al., 1984). This convinces us that the results obtained with the Cu^{2+}–enzyme may be extrapolated to other activator metals as well. Furthermore, the similarities between the data obtained for the spinach enzyme and the *R. rubrum* enzyme suggest that these structurally quite different enzymes have similar catalytic mechanisms.

ACKNOWLEDGMENTS

We are grateful to Professor T. Vänngård, Dr. R. Aasa, T. Nilsson, and Ö. Hansson for valuable discussions concerning the interpretation of the EPR data. We also thank Gerd Holmquist for help in preparation of the manuscript.

Registry No. RuBP, 24218-00-6; CABP, 27442-42-8; 3-PGA, 820-11-1; 2-PGA, 2553-59-5; CRBP, 29843-30-9; Cu, 7440-50-8; phosphohydroxypyruvic acid, 3913-50-6; fructose 1,6-bisphosphate, 488-69-7; 6-phosphogluconic acid, 921-62-0; ribose 5-phosphate, 4300-28-1; sedoheptulose 1,7-bisphosphate, 815-91-8; ribulose-1,5-

bisphosphate carboxylase/oxygenase, 9027-23-0.

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