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Ribulose-1,5-bisphosphate Carboxylase/Oxygenase Incubated with Cu^{2+} and Studied by Electron Paramagnetic Resonance Spectroscopy[†]

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ABSTRACT: Ribulose-1,5-bisphosphate carboxylase/oxygenase binds Cu^{2+} at a specific site when stoichiometric amounts of Cu^{2+} are added at saturating concentrations of HCO_3^-. The presence of HCO_3^- (CO_2) is necessary to obtain specific binding of Cu^{2+}. The activating metals Mg^{2+} and Co^{2+} compete with Cu^{2+}, indicating a common site on the enzyme for these metals. Electron paramagnetic resonance (EPR) proves that at least one nitrogen ligand is involved in the binding of Cu^{2+} at this site. When ribulose 1,5-bisphosphate is added to the Cu^{2+}-incubated enzyme, a tight enzyme–Cu^{2+}–ribulose 1,5-bisphosphate complex is formed. In this ternary complex only oxygen atoms are directly coordinated to Cu^{2+} as seen by its characteristic EPR spectrum. A similar but distinct spectrum is obtained if 2-carboxyarabinitol 1,5-bisphosphate, an analogue to the six-carbon intermediate in the RuBP carboxylase reaction, is added to the Cu^{2+}-incubated enzyme. These results indicate that a metal–substrate complex is formed at the active site of the enzyme. A function for the metal in catalysis is thus implied.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) is a metal-activated enzyme that catalyzes two reactions (Calvin & Massini, 1952; Andrews et al., 1973):

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

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

The active form of the enzyme contains bound CO_2 and a metal ion. The CO_2 molecule is bound as a carbamate and is stabilized by the metal ion (Lorimer et al., 1976; Lorimer & Miziorko, 1980). There is, however, no experimental evidence for a direct coordination of the metal to the carbamate.

The enzyme that in vivo is activated by Mg^{2+} has also been shown to be catalytically active in the presence of Mn^{2+}, Co^{2+}, Ni^{2+}, and Fe^{2+} (Christeller, 1981). Interaction with other transition metals has not been seriously studied, probably because they support very little activity.

In catalysis a molecule of CO_2, different from the CO_2 in the activating process, or O_2 reacts with RuBP[1] (reactions 1 and 2) (Lorimer, 1981). So far, it has not been proved that the metal is involved in the catalytic reactions, but some indications for a direct participation exist. The metal and the catalytic CO_2 are close enough to suggest a role for the metal in catalysis (Miziorko & Mildvan, 1974). The F^- inhibition of the Mg^{2+}- and Mn^{2+}-activated enzymes (Nilsson & Brändén, 1983) and the drastic drop of K_i for O_2 for the Mn^{2+}-activated enzyme, compared to the Mg^{2+}-activated enzyme (Christeller, 1981), indicate that the metal ion participates directly in catalysis.

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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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Transition metals with measurable spectroscopic properties are often used as probes for the mechanism and structure of metal-activated enzymes (Malmström, 1972). For certain paramagnetic metals, electron paramagnetic resonance (EPR) spectroscopy is particularly useful. An EPR spectrum is a sensitive "fingerprint" of the metal environment, and a change here is often reflected in the EPR spectrum. Mn^{2+}, Co^{2+}, and Fe^{2+}, which activate RuBP carboxylase/oxygenase, are all paramagnetic and have distinct EPR spectra. Another paramagnetic metal ion is Cu^{2+}, and several proteins that contain bound copper necessary for their catalytic function have been extensively studied by the EPR technique (Spiro, 1981).

In EPR spectroscopy the microwave absorption of a paramagnetic sample, placed in an external magnetic field, is studied. When the magnetic field is varied, absorptions are obtained at distinct magnetic fields. The absorptions are better resolved if the spectrum is derivated. Therefore, the first derivative absorption of the EPR spectrum is presented. In a spectrum of a Cu^{2+} complex, two distinct regions are recognized. At the low-field part of the spectrum, four hyperfine lines, which are characteristic for a Cu^{2+} complex, are easily detectable. In the high-field part of the spectrum, four lines are also expected, but these lines are normally not resolved.

The appearance of the EPR spectrum is dependent on the ligands that are directly coordinated to Cu^{2+}. Normally only oxygen or nitrogen atoms are directly coordinated to Cu^{2+} in a protein molecule, although sulfur sometimes is found as a ligand (Spiro, 1981). If the coordinated atom has a nuclear spin, for instance, ^{14}N ($I = 1$), an interaction with the electron spin of Cu^{2+} occurs. This results in a splitting of the hyperfine lines. In the case of nitrogen ligands, the splitting is usually not resolved, and a Cu^{2+} complex with one or more nitrogen ligands therefore gives rise to rather broad hyperfine lines. At the high-field part of the spectrum, the splitting may be easier to observe and is, when detectable, an indication that the Cu^{2+} complex is homogeneous. In an EPR spectrum, obtained from a mixture of different Cu^{2+} complexes, nitrogen hyperfine splitting will hardly be observed since the number of lines will be too large.

^{16}O has no nuclear spin, in contrast to ^{14}N, and does not give rise to hyperfine splitting. A Cu^{2+} complex with only ^{16}O atoms as direct ligands may therefore have narrow EPR hyperfine lines.

Here we show, by the use of EPR, that Cu^{2+} binds at the activator metal binding site of the RuBP carboxylase/oxygenase molecule. In presence of RuBP, a tight enzyme–Cu^{2+}–RuBP complex is formed, in which only oxygen atoms are coordinated to the Cu^{2+} ion as seen from its EPR spectrum. Binding of the transition-state analogue 2-carboxyarabinitol 1,5-bisphosphate (CABP) gives a complex with a similar but distinct EPR spectrum. These findings indicate that the activating metal ion binds close to the RuBP site in the enzyme molecule. The metal might therefore be essential for catalysis as well as for activation of the 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

Materials. Tris(hydroxymethyl)aminomethane was obtained from Merck and Co. RuBP, D-3-PGA,[1] dithiothreitol, and *N*-(2-hydroxyethyl)piperazine-*N'*-propanesulfonic acid (HEPPS) were purchased from Sigma Chemical Co. Sephacryl S-300, Sephadex G-25, and DEAE-Sepharose CL-6B were obtained from Pharmacia Fine Chemicals. $NaH^{14}CO_3$ was purchased from Amersham and ^{63}CuO from Oak Ridge Laboratory. Nucleosil 10 SB was obtained from Macherey-Nagel, and CABP was prepared as described in Pierce et al. (1980). All other chemicals were of analytical grade.

Enzyme Purification. RuBP carboxylase/oxygenase was purified from spinach as described earlier (Nilsson & Brändén, 1983). The specific activity of the enzyme was 1.5 μmol of CO_2 fixed min^{-1} mg^{-1} at 25 °C and pH 8.3.

Protein Determination. The concentration of RuBP carboxylase/oxygenase was calculated from the absorption at 280 nm and $A_{280}^{1\%} = 16.4$ (Paulsen & Lane, 1966).

High-Pressure Liquid Chromatography. High-pressure liquid chromatography was used for identification of phosphorus compounds in reaction mixtures. An aliquot of the reaction mixture was treated with $HClO_4$ (final concentration 0.5 M) and then neutralized with KOH. The precipitate was removed by centrifugation, and the supernatant was analyzed. Phosphates were separated on an anion-exchange column (Nucleosil SB, 10 μm, 4.6 × 250 mm). The mobile phase was sodium perchlorate at pH 2.8 (adjusted with $HClO_4$) containing 0.25 mM citric acid. Elution was carried out with 0.02 M $NaClO_4$ for 30 min, followed by a gradient of 0.02–0.075 M $NaClO_4$ for 30 min. The flow was 0.8 mL min^{-1}. Eluted compounds were detected with a phosphate-sensitive post-column reaction detector, which is based on an automatic method for total phosphate in seawater (Grasshoff, 1976) and will be described in detail elsewhere.

Radioactive Measurements. Radioactivity was measured with a Packard 3255 Tri-Carb liquid scintillation spectrometer.

Atomic Absorption Spectrometry. Atomic absorption measurements were made with a Perkin-Elmer 2380 spectrophotometer.

Anaerobic EPR Experiments. The technique used for anaerobic EPR experiments has been described earlier (Fee et al., 1969).

EPR Measurements. EPR spectra were recorded at 77 K and 9.1 GHz in a Varian E-3 spectrometer using a microwave power of 10 mW. The amount of EPR-detectable copper was determined by double integration of the 9.1-GHz spectra, and corrections for the different *g* factors were applied (Aasa & Vänngård, 1975).

Results

Binding of Cu^{2+} to RuBP Carboxylase/Oxygenase. When one Cu^{2+} per enzyme protomer was added to the enzyme in the presence of HCO_3^-, the EPR spectrum seen in Figure 1B was obtained. As indicated by the nitrogen hyperfine structure at the high-field part of the spectrum (Figure 1D), most of the EPR-detectable Cu^{2+} is bound at a single site on the enzyme where at least one nitrogen atom is coordinated to the Cu^{2+} ion. If HCO_3^- was omitted in this experiment, an EPR spectrum with broad and mixed hyperfine structure was obtained (EPR spectrum not shown), indicating that Cu^{2+} under this condition binds at several different sites on the enzyme. In some experiments enzyme was activated with HCO_3^- and Mg^{2+} or Co^{2+} prior to the addition of Cu^{2+} in order to examine the influence of these metal ions on the binding of Cu^{2+}. Enzyme (0.6 mM protomers in 50 mM HEPPS buffer at pH 8.0) was preincubated with Mg^{2+} (50 mM) and HCO_3^- (120 mM) for 10 min before the addition of Cu^{2+} (0.5 mM). After 30 min at room temperature, the samples were frozen in EPR tubes at liquid nitrogen. The changes in the EPR spectra due to the presence of Mg^{2+} were analyzed at the low-field part of the spectra. The amount of specifically bound Cu^{2+} was ca. 50% of that obtained in the absence of Mg^{2+}. Similarly, Co^{2+} (5 mM) reduced the specific binding of Cu^{2+} to ca. 25%.

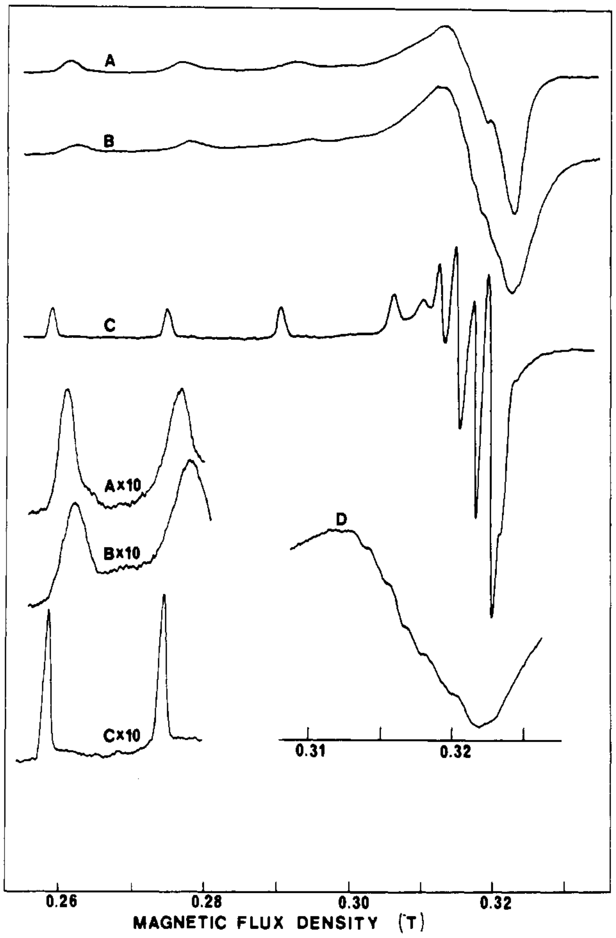

FIGURE 1: EPR spectra of Cu^{2+}-incubated RuBP carboxylase in the absence and presence of RuBP. (A) Control experiment. The EPR spectrum of $^{63}CuCl_2$ (0.4 mM) in 50 mM HEPPS buffer at pH 8.0 containing $NaHCO_3$ (25 mM) and RuBP (8 mM). The spectrometer gain was 4×10^4. (B) To 250 μL of enzyme (41 mg/mL in 50 mM HEPPS buffer at pH 8.0 containing 25 mM HCO_3^-) was added 10 μL of $^{63}CuCl_2$ (12.5 mM). After 30 min at room temperature the solution was frozen in an EPR tube at liquid nitrogen. The spectrometer gain was 2×10^5. (C) To 250 μL of enzyme (41 mg/mL in 50 mM HEPPS buffer at pH 8.0) 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 solutions were made anaerobic before mixing. The spectrometer gain was 1×10^5. The EPR parameters of the spectrum are $g_\parallel = 2.301$, $g_\perp = 2.055$, $A_\parallel = 15.75$ mT, and $A_\perp = 2.39$ mT. (D) The high-field part of the spectrum seen in (B). The modulation amplitude was 1 mT in spectra A–C and 0.5 mT in spectrum D.

Mg^{2+} and Co^{2+} thus seem to compete with Cu^{2+} for the same site of the enzyme.

Effect of RuBP Added to the Cu^{2+}-Incubated RuBP Carboxylase/Oxygenase. Figure 1C shows the characteristic EPR spectrum obtained when RuBP was added to enzyme incubated with stoichiometric amounts of Cu^{2+} in the presence of HCO_3^- and under anaerobic conditions. This spectrum is distinct from the spectrum of Cu^{2+} in buffer only (Figure 1A) as well as from the spectrum obtained when Cu^{2+} was added to the enzyme in the absence of RuBP (Figure 1B). Particularly noticeable are the narrow hyperfine lines observable at the low-field part of the EPR spectrum. The spectrum seen in Figure 1C was obtained by using isotopically pure $^{63}Cu^{2+}$. The natural isotopic composition of copper (70% ^{63}Cu, 30% ^{65}Cu) yields a more complex spectrum due to the different hyperfine coupling constants of the two isotopes.

The presence of HCO_3^- was necessary for the formation of the EPR spectrum seen in Figure 1C. Cu^{2+} added to a nonactivated enzyme–RuBP complex (Jordan & Chollet, 1983) or RuBP added to Cu^{2+}-incubated enzyme in the absence of HCO_3^- did not give rise to the spectrum seen in Figure 1C.

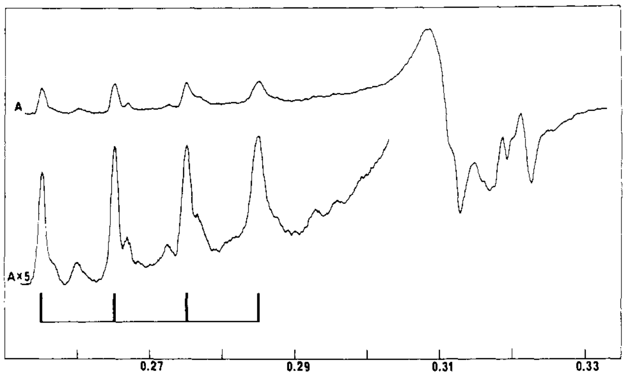

FIGURE 2: EPR spectrum obtained when CABP was added to Cu^{2+}-incubated enzyme. To 250 μL of enzyme (70 mg/mL in 50 mM HEPPS buffer at pH 8.0) were added 20 μL of $NaHCO_3$ (0.5 M) and 15 μL of $^{63}CuCl_2$ (12.5 mM). After 3 min 25 μL of CABP (to a concentration of 1–1.5/active site) was added; 5 min later the solution was frozen in an EPR tube at liquid nitrogen. The spectrometer gain was 1×10^5 and the modulation amplitude was 0.5 mT. The following EPR parameters were obtained: $g_\parallel = 2.405$; $A_\parallel = 10.3$ mT.

Stability and Composition of the Enzyme–Cu^{2+} Complex. The complex formed between Cu^{2+}-incubated enzyme and RuBP (Figure 1C) still contained the same amount and type of EPR-detectable copper after a gel filtration on a Sephadex G-25 column equilibrated with 50 mM HEPPS buffer at pH 8.3. After gel filtration the complex could be stored for 24 h at 4 °C without any decrease of the EPR signal, which indicates a remarkable stability.

A solution of the gel-filtered enzyme–Cu^{2+} complex, formed in the presence of RuBP, was analyzed by high-pressure liquid chromatography for phosphorus compounds as described under Materials and Methods. Only RuBP was detected. The ratio of RuBP to EPR-detectable copper was 1, indicating that a ternary enzyme–Cu^{2+}–RuBP complex gives the EPR spectrum seen in Figure 1C. When the same gel-filtered solution was analyzed for copper by atomic absorption spectrophotometry, it was found that all added copper remained bound to the enzyme. Of that, one-third was EPR detectable. The remaining part of the copper, which is not detectable by EPR and therefore not paramagnetic, must be bound as Cu^+. The nature of this binding site is not known.

A gel filtration of an enzyme–Cu^{2+}–RuBP complex formed in the presence of $H^{14}CO_3^-$ showed that $^{14}CO_2$ was not retained.

Effect of CABP. When stoichiometric amounts of CABP were added to the Cu^{2+}-incubated enzyme, an EPR spectrum with narrow hyperfine lines was obtained (Figure 2). This spectrum is similar but distinct from the spectrum obtained when RuBP was added (Figure 1C). No loss of EPR-detectable Cu^{2+} was obtained if the enzyme–Cu^{2+}–CABP complex was gel filtered, indicating that the complex is tight.

In order to test the specificity of the Cu^{2+} binding, an inert enzyme–CO_2–Mg^{2+}–CABP complex was made according to Hall et al. (1981). Enzyme (0.39 mM protomer) was incubated for 1 h at 25 °C with 20 mM HCO_3^-, 11 mM Mg^{2+}, 0.8 mM CABP, and 50 mM HEPPS buffer at pH 8.0. The enzyme was thereafter gel filtered on a Sephadex G-25 column equilibrated with 50 mM HEPPS buffer containing 20 mM HCO_3^- at pH 8.0. To the gel-filtered enzyme–CO_2–Mg^{2+}–CABP complex (0.19 mM protomer) was added Cu^{2+} (to a final concentration of 0.19 mM) prior to the addition of CABP (0.40 mM) or D-3-PGA (5 mM). No narrow EPR hyperfine

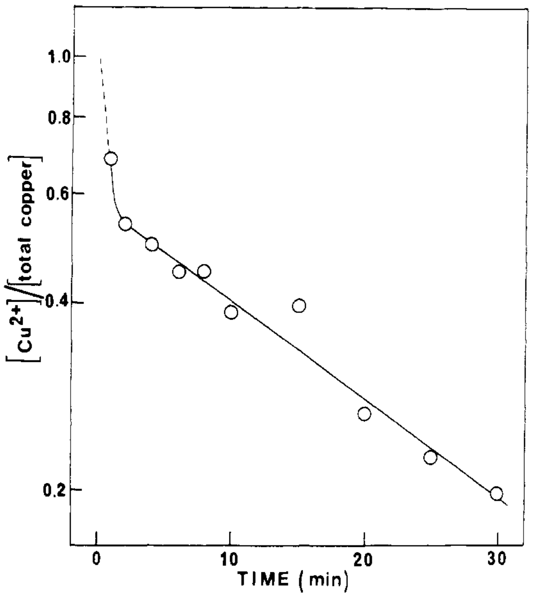

FIGURE 3: Time course for the reduction of Cu^{2+} added to the enzyme. To 4.0 mL of enzyme (15 mg/mL) were added 100 μL of NaHCO$_3$ (1 M) and 35 μL of ^{63}CuCl$_2$ (18.5 mM). Aliquots were taken at different times and frozen at liquid nitrogen in EPR tubes. The amount of Cu^{2+} was determined from the EPR spectra.

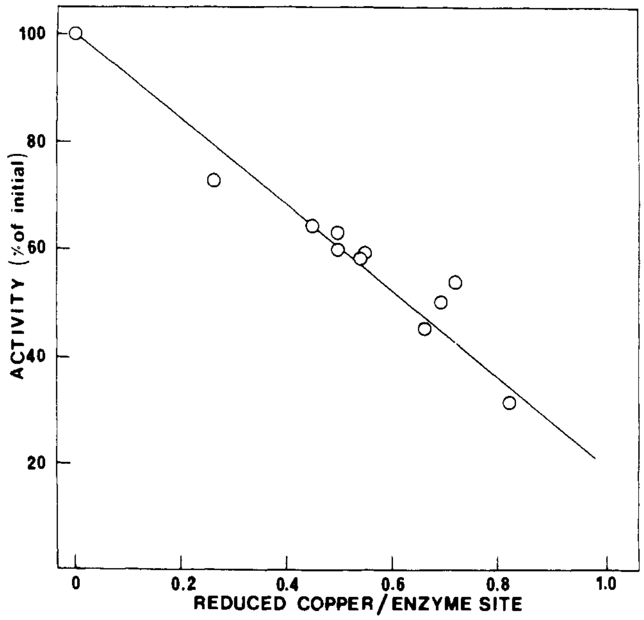

FIGURE 4: Correlation between activity and EPR-detectable Cu^{2+}. Aliquots were taken from the same enzyme solution as in Figure 3. At the same time as a sample for EPR was frozen, an aliquot was added to 50 mM HEPPS buffer at pH 8.0 containing Mg^{2+} (50 mM) and HCO$_3^-$ (50 mM). After 30 min at 25 °C the activity of the enzyme was measured. Initial activity (100%) is the normal activity of the enzyme in the absence of Cu^{2+} but otherwise identical conditions with that above. The amount of EPR-detectable Cu^{2+} was determined from the EPR spectra.

lines could be observed for the two samples in contrast to those seen in Figure 2 and Figure 3 of Brändén et al. (1984b) when CABP or D-3-PGA was added to Cu^{2+}-incubated enzyme.

Stoichiometry of Enzyme-Bound Cu^{2+}. At the slightly alkaline pH of a RuBP carboxylase/oxygenase assay, precipitation of Cu(OH)$_2$ is a problem. However, HCO$_3^-$ increases the solubility of Cu^{2+} in the assay. The solubility of Cu^{2+} could be measured by quantitating the maximum amount of Cu^{2+} dissolved in the assay by integration of EPR spectra. It was found that in an assay containing 50 mM HEPPS buffer at pH 8.0 and 25 or 50 mM HCO$_3^-$ the solubilities of Cu^{2+} were 300 and 550 μM, respectively.

DTT must be completely removed from the enzyme because it rapidly reduces Cu^{2+}. When Cu^{2+} (0.5 mM) was added to 50 mM HEPPS buffer at pH 8.3, containing 50 mM HCO$_3^-$ and 1 mM DTT, the EPR signal of Cu^{2+} disappeared within 30 s.

In order to investigate the activator properties of Cu^{2+}, we therefore added stoichiometric amounts of Cu^{2+} (one Cu^{2+} per protomer) or less in the absence of DTT. The EPR-detectable Cu^{2+} was, under these conditions, bound to the enzyme as seen from the EPR spectrum in the presence of enzyme, which is different from the spectrum in buffer only (Figure 1A,B). However, some of the added Cu^{2+} was nevertheless reduced, probably by SH groups at the protein surface. When Cu^{2+} (final concentration 0.15 mM) was added to RuBP carboxylase/oxygenase (15 mg/mL in 50 mM HEPPS buffer at pH 8.0 containing 50 mM HCO$_3^-$), the time-dependent reduction of Cu^{2+} could be followed by the disappearance of the EPR signal as shown in Figure 3.

The part of the added Cu^{2+} that was reduced (Figure 3) must have been accompanied by oxidation of enzyme molecules. We investigated if this oxidation resulted in an inactive enzyme. In an experiment aliquots were taken at different times after Cu^{2+} had been added to the enzyme solution. The amounts of oxidized and reduced copper were determined by EPR (oxidized copper = EPR-detectable Cu^{2+}; reduced copper = added copper − EPR-detectable Cu^{2+}). The activity at each point was measured after aliquots had been activated in buffer containing Mg^{2+} (50 mM), 5 mM EDTA, and HCO$_3^-$ (50 mM) for 30 min at 25 °C. When the Cu^{2+}-incubated enzyme was added to the activating buffer, the time-dependent loss of enzyme activity seen in Figure 3 stopped. This was tested in a separate experiment.

Figure 4 shows the correlation between enzyme activity and the amount of reduced copper. It is clearly seen that reduction of a certain amount of copper causes an equal loss of enzyme activity. The remaining oxidized copper, which was shown to be enzyme bound, therefore did not give rise to an inactivation of the enzyme but is simply replaced by Mg^{2+} in the activating Mg^{2+}-containing buffer. The enzyme–Cu^{2+}–RuBP complex obtained upon addition of RuBP is thus formed from an intact enzyme.

The EPR spectrum in Figure 1C, which was obtained when a stoichiometric amount of Cu^{2+} and excess RuBP were added to the enzyme, constitutes only about 30% of the added Cu^{2+}. In order to increase the amount of this Cu^{2+} complex, enzyme was also incubated with higher concentrations of Cu^{2+} before the addition of RuBP. At most, 0.8 specific Cu^{2+}/protomer could be obtained. However, under these conditions nonspecific binding of Cu^{2+} also occurred, which complicated interpretations of EPR spectra.

Activity of the Cu^{2+}-Incubated RuBP Carboxylase/Oxygenase. Cu^{2+} (final concentration 0.12 mM) was added to a solution of RuBP carboxylase/oxygenase (0.13 mM protomer) containing NaH^{14}CO$_3$ (10 mM) in 50 mM HEPPS buffer at pH 8.3. After 10-min incubation time enzyme was added to an assay containing RuBP (1.2 mM) and NaH^{14}CO$_3$ (10 mM) at pH 8.3 and 25 °C. At different times, from 1 to 20 min, samples were taken and denatured with 2 M HCl. The samples were then taken to dryness at 90 °C for 2 h, and the radioactivity was determined. A control experiment was performed in the absence of Cu^{2+}. A time-dependent inactivation of the enzyme was observed. However, the total amount of fixed CO$_2$, measured after a 10-min reaction time, was 40 nmol mg^{-1}, corresponding to about two turnovers per enzyme protomer. From Figure 3 it is seen that only 40% of the added copper remains oxidized 10 min after its addition to the enzyme solution. Figure 4 also shows that activity is correlated with the amount of remaining Cu^{2+}. Therefore, the activity of the Cu^{2+}-containing enzyme is approximately 10 nmol of fixed CO_2 min^{-1} (mg of Cu^{2+}-containing protomer)$^{-1}$. After 10 min this corresponds to five turnovers per Cu^{2+}-containing protomer.

Discussion

The use of Cu^{2+} as a metal probe for RuBP carboxylase/oxygenase has here been shown to be very fruitful although Cu^{2+} supports activity poorly. The paramagnetic property of Cu^{2+} makes it possible to observe different enzyme–Cu^{2+} complexes, under various conditions, and the EPR spectra also provide some information about the nature of the ligands in these complexes.

The results presented here show that Cu^{2+} is bound at a single site on the intact enzyme molecule when stoichiometric amounts of Cu^{2+} are added to an enzyme solution containing HCO_3^-. This is based on the observation of the nitrogen hyperfine structure at the high-field part of the EPR spectrum (Figure 1D). Such a structure would hardly be observed if Cu^{2+} was bound at several different sites on the enzyme molecule. The effects of Mg^{2+} and Co^{2+} on the binding of Cu^{2+} and the inability to achieve specific Cu^{2+} complexes using the inert enzyme–CO_2–Mg^{2+}–CABP complex suggest that Cu^{2+} in the presence of HCO_3^- binds at the same site as other activating metal ions.

The EPR spectra of the enzyme–Cu^{2+} complex (Figure 1B,D) also tell us that nitrogen atom(s) is (are) directly coordinated to the metal in this complex. As histidine, lysine, and arginine residues have been shown to play essential roles for the function of the enzyme (Robison & Tabita, 1979; Saluja & McFadden, 1980; Hartman et al., 1981), one of these side chains may be involved in the binding of the metal. A nitrogen atom has been shown to be close to the metal in the Cr^{2+}-incubated enzyme (Miziorko et al., 1982).

When an excess of RuBP was added to the Cu^{2+}-incubated enzyme, a tight enzyme–Cu^{2+}–RuBP complex was formed that has an EPR spectrum with unusually narrow hyperfine lines (Figure 1C). A similar spectrum has been observed for Cu^{2+}–acetyl acetonate in which all ligands are oxygen atoms (Vänngård, 1972). Narrow hyperfine lines have also been observed for Cu^{2+} bound to apo yeast enolase in the presence of the substrate 2-phosphoglycerate (Dickinson et al., 1980) and for Cu^{2+} in yeast inorganic pyrophosphatase (B. Cooperman, personal communication). For these two enzymes oxygen atoms are suggested to be the only ligands to the Cu^{2+} ions. ^{16}O has no nuclear spin, and therefore narrow EPR hyperfine lines are expected to be formed for a complex with only oxygen atoms coordinated to the Cu^{2+} ion. However, if ^{14}N atom(s) ($I = 1$) is (are) coordinated to the metal, it will give rise to a broadening effect due to the splitting of the hyperfine lines. Theoretically, it would be possible to have narrow hyperfine lines also in the presence of nitrogen ligands (if the coupling constant is very small), but this is a highly improbable situation (T. Vänngård, personal communication). The narrow hyperfine lines in the EPR spectrum of the enzyme–Cu^{2+}–RuBP complex are therefore compatible only with oxygen atoms coordinated to Cu^{2+}. The nitrogen atom(s) coordinated to Cu^{2+} in the enzyme–Cu^{2+} complex seen in Figure 1B must thus have been displaced by oxygen atoms after the binding of RuBP. The simplest explanation of this result is that RuBP is directly coordinated to Cu^{2+} and provides the new oxygen ligand(s). Since Cu^{2+} was shown to compete with Mg^{2+} and Co^{2+} for the same site, this result indicates that the metal ion site and the RuBP site are close to each other in the enzyme molecule. Although a direct coordination of RuBP to the metal ion, as indicated here, has not been shown for the Mg^{2+}-activated enzyme, a change in the metal environment induced by RuBP binding has earlier been inferred from the F^- inhibition kinetics of the Mg^{2+}-activated enzyme (Nilsson & Brändén, 1983).

A tight enzyme–Cu^{2+} complex was also formed when CABP was added to the Cu^{2+}-incubated enzyme (Figure 2). The high $g_\parallel$ value ($g_\parallel = 2.401$), the rather small hyperfine splitting constant ($A_\parallel = 10.3$ mT), and the narrow hyperfine lines indicate that the enzyme–Cu^{2+}–CABP complex is a distorted complex with only oxygen atoms directly coordinated to the Cu^{2+} ion. One or more of these oxygen ligands may originate from CABP similarly to what we suggest for the ternary enzyme–Cu^{2+}–RuBP complex. A direct coupling between oxygen atoms from CABP and the metal ion has been shown to exist for the Mn^{2+}-activated enzyme from *Rhodospirillum rubrum* when ^{17}O-enriched CABP is used (Miziorko, 1984).

The present work provides additional evidence for a role for the metal in catalysis of RuBP carboxylase/oxygenase. Other results, obtained at low concentrations of RuBP and under aerobic conditions, indicate the existence of a transient enzyme–Cu^{2+} complex dependent on the concentration of O_2. The nature of this complex and its relation to the oxygenase reaction will be discussed in the following paper (Brändén et al., 1984b).

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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$_2$[†]

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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 & Brändên, 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 (Brändên 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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