Electron Spin Resonance Studies of Ribulosebisphosphate Carboxylase: Identification of Activator Cation Ligands†

Henry M. Miziorko* and Roger C. Sealy

ABSTRACT: Ribulosebisphosphate carboxylase (RuBP carboxylase) forms a stable model complex containing stoichiometric amounts of enzyme sites, activator CO_2, divalent activator cation, and the transition-state analogue carboxyarabinitol bisphosphate (CABP). Incorporation of Mn^{2+} in the model complex permits investigation of the environment of the activator cation by electron spin resonance (ESR) techniques. Measurements at 9 GHz on the Mn^{2+}-containing complex prepared by using dimeric *Rhodospirillum rubrum* enzyme produce a spectrum which indicates that the cation is bound in an anisotropic environment. Measurements at 9 GHz on the spinach enzyme model complex produce a spectrum in which several of the fine structure transitions are obvious. In contrast, the spectrum produced from Mn^{2+} bound to *R. rubrum* enzyme exhibits an intense powder pattern for the central fine structure transition; the other four fine structure transitions produce powder patterns that are inhomogeneously broadened and therefore are not as apparent. Low-temperature measurements at high field (35 GHz) result in substantially simplified spectra. The spectrum of Mn^{2+} bound to the *R. rubrum* enzyme shows less fine structure than the spectrum of Mn^{2+} bound in the octameric spinach enzyme complex, where substantial hyperfine splitting is resolved in three of the five fine structure transitions. Measurements at 35 GHz on Mn^{2+} bound in the dimeric *R. rubrum* enzyme complex produce spectra in which only the central fine structure transition produces a prominent signal. However, these samples are characterized by several narrow spectral features which permit investigation of the identity of Mn^{2+} ligands by ^{17}O perturbation techniques. Preparation of the *R. rubrum* RuBP carboxylase model complex in ^{17}O-enriched water results in a sample which exhibits an obviously broadened 35-GHz Mn^{2+} spectrum in comparison to unenriched samples. Removal of $H_2{}^{17}O$ by gel filtration abolished the spectral broadening, indicating that the Mn^{2+}-coordinated water molecules can slowly exchange. No spectral broadening was detectable due to ^{17}O in the carbamate oxygens derived from activator $C^{17}O_2$. NMR relaxation rate measurements at 24.3 MHz demonstrate that stoichiometric amounts of carboxyarabinitol bisphosphate eliminate enhancement of the proton relaxation rate observed in ternary enzyme–CO_2–Mn^{2+} complexes prepared by using dimeric *R. rubrum* enzyme. This observation, coupled with results of the $H_2{}^{17}O$ ESR experiments, is compatible with the suggestion that the water molecules which coordinate directly to bound Mn^{2+} are nonexchangeable on an NMR time scale but can be displaced by solvent water within 1–2 h. Carboxyarabinitol bisphosphate was selectively enriched with ^{17}O in the carboxyl group or in the oxygen on C-2. Mn^{2+}-containing complexes prepared with either of the ^{17}O-enriched analogues produced spectra which were broadened in comparison to matched ^{16}O controls. Thus, Mn^{2+} coordinates directly to CABP, arguing for the participation of cation in the catalytic process.

Ribulosebisphosphate carboxylase (RuBP carboxylase)[1] catalyzes the reaction of its sugar phosphate substrate with CO_2 to form two molecules of 3-phosphoglycerate (Quayle et al., 1954; Weissbach et al., 1954) and with O_2 to form phosphoglycolate and phosphoglycerate (Bowes et al., 1971; Andrews et al., 1973). Both reactions are dependent upon a divalent cation. Conversion of the isolated enzyme to a catalytically functional form requires the presence of a divalent cation, as well as an activator CO_2 molecule (Lorimer et al., 1976). Direct involvement of the cation in both enzyme activation (Laing & Christeller, 1976) and catalysis (Miziorko & Mildvan, 1974) has been invoked, and a reasonable chemical rationale has been offered to support these hypotheses. However, little evidence is available to unequivocally demonstrate the role of the cation. ^{13}C NMR studies on the spinach enzyme indicated proximity between the bound cation and a rapidly exchanging species of CO_2 that was interpreted to be the substrate (Miziorko & Mildvan, 1974); the data suggested a direct role for the metal in catalysis. Subsequently, it was demonstrated that two CO_2 molecules are bound per active site (Miziorko, 1979; Lorimer, 1979), raising some question concerning the identity of the CO_2 molecule which interacts with the cation. Since activator CO_2, upon binding to enzyme, forms a carbamate (Lorimer & Miziorko, 1980) which should not be in rapid exchange with CO_2 in the aqueous medium, the postulate of a cation–substrate–CO_2 interaction remained a reasonable one. A role for the cation in catalysis is also supported by reports that the partioning of RuBP between carboxylation and oxygenation reactions is dependent upon which cation is supplied to the enzyme (Wildner & Henkel, 1979; Christeller & Laing, 1979; Jordan & Ogren, 1981).

Direct observation of the cation activator has been accomplished in ESR experiments (Miziorko & Sealy, 1980) using the model quaternary complex composed of stoichiometric amounts of RuBP carboxylase, activator CO_2, Mn^{2+}, and the transition-state analogue carboxyarabinitol bisphosphate (CABP). The ESR spectra suggest that there is marked distortion of the coordination sphere of the cation bound in the spinach enzyme model complex, probably due to unusual

† From the Departments of Biochemistry (H.M.M.) and Radiology (R.C.S.), The Medical College of Wisconsin, Milwaukee, Wisconsin 53226. *Received June 8, 1983.* This work was supported in part by grants from the U.S. Department of Agriculture Competitive Research Grants Program (79-CRCR-1-0339) and the National Institutes of Health (P41 RR01008). H.M.M. is the recipient of U.S. Public Health Service Research Career Development Award AM-00645.

[1] Abbreviations: RuBP, ribulose bisphosphate; CABP, carboxyarabinitol bisphosphate; ESR, electron spin resonance; $1/T_1$, longitudinal relaxation rate; M^{2+}, divalent cation; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/84/0423-0479$01.50/0

inner-sphere liganding. Amino acids, activator CO_2, CABP, and solvent water molecules could coordinate to the bound activator cation, which is expected to accomodate six inner-sphere ligands. Recent work suggests that, in model quaternary complexes formed by using both octameric spinach and dimeric *Rhodospirillum rubrum* enzymes, protein does, in fact, coordinate directly to the cation (Miziorko et al., 1982). This report describes the further application of the ESR approach to study RuBP carboxylase. Observation of relatively narrow spectral features in studies performed at 35 GHz using samples prepared with *R. rubrum* enzyme prompted investigation of whether superhyperfine coupling between bound Mn^{2+}- and ^{17}O-enriched components of the quaternary complex occurs. Such interactions are detectable, and their observation permits a substantial refinement in our understanding of the function of the activator cation of RuBP carboxylase. A preliminary account of this work has appeared (Miziorko & Sealy, 1983).

Experimental Procedures

Materials. *R. rubrum* RuBP carboxylase was the generous gift of Dr. F. C. Hartman. Spinach leaf RuBP carboxylase was prepared as described by Paulsen & Lane (1966). Enzyme activity was assayed by standard radioisotopic (Lorimer et al., 1977) or spectrophotometric (Andrews et al., 1973) procedures. Protein concentration was estimated spectrophotometrically by using extinction coefficients previously reported (Paulsen & Lane, 1966; Stringer et al., 1981). RuBP was purchased from Sigma. [^{16}O]CABP was synthesized as described by Pierce et al. (1980). Sephadex was supplied by Pharmacia. $NaH^{14}CO_3$ and $K^{14}CN$ were purchased from New England Nuclear. Chelex 100 resin was obtained from Bio-Rad Laboratories and was used to prepare metal-free reagents and buffers. Ultrapure Tris base and urea were purchased from Schwarz/Mann. $H_2{}^{17}O$ (45% enriched) was obtained from Miles/Yeda. All other reagents were of the highest purity commercially available.

Methods. Enzyme was desalted before use by Sephadex G-25 chromatography in 50 mM Tris-HCl, pH 7.8. Activation of enzyme with CO_2 and cation, as well as formation of the quaternary complex upon incubation with CABP, was performed as previously described (Miziorko, 1979; Miziorko et al., 1982). Two millimolar $MnCl_2$ was used to form all quaternary complexes, which were subsequently isolated free of unbound components by Sephadex G-75 chromatography. In the $H_2{}^{17}O$ experiment, the only procedure in which the quaternary complex was not G-75 purified, a ratio of 0.84 Mn^{2+} per enzyme site was employed to assure that no free cation would remain to obscure the ESR measurements.

Preparation of a $C^{17}O_2$ species was accomplished by incubating $KHCO_3$ in an $H_2{}^{17}O$-enriched solution for 2 h at 30 °C in the presence of an excess of carbonic anhydrase. Subsequent additions of enzyme, Mn^{2+}, and CABP were made from concentrated solutions so that minimum isotopic dilution would occur. Final isotopic enrichment in the $H_2{}^{17}O$ and $C^{17}O_2$ experiments was 42%.

Enrichment of the carboxyl oxygens of CABP was accomplished by lactonizing purified CABP and subsequently delactonizing in $H_2{}^{17}O$. The solution was then adjusted to pH 1 and incubated at 22 °C for 90 h. The sample was neutralized with KOH (in $H_2{}^{17}O$). $H_2{}^{17}O$ was then removed by bulb to bulb distillation. The ^{17}O-enriched CABP was dissolved in $H_2{}^{16}O$ prior to use. Aliquots of the solution were treated with alkaline phosphatase and pertrimethylsilylated prior to mass spectroscopic analysis (Hewlett Packard Model 5985) in order to verify isotopic enrichment (Biemann, 1962). Specific labeling of the carboxyl oxygens (30% enrichment) was achieved. In other experiments, which produced results similar to those reported here, the carboxyl oxygens of CABP were labeled by two cycles of lactonization–delactonization in $H_2{}^{17}O$; 32% enrichment was achieved by this approach. The alcoholic oxygen on C-2 of CABP was enriched with ^{17}O by hydrolyzing the cyanohydrin prepared from KCN and RuBP in $H_2{}^{17}O$. ^{17}O in the carboxyl oxygens was selectively depleted by four cycles of lactonization–delactonization in $H_2{}^{16}O$. Mass spectroscopic analysis of the analogue after dephosphorylation and pertrimethylsilylation indicated specific labeling of the alcoholic oxygen with 45% enrichment.

Binding stoichiometries and the exchange-inert nature of the isolated quaternary complexes were verified by following the ^{14}C radioactivity incorporated by using labeled components to form the quaternary complexes. In the $C^{17}O_2$, $H_2{}^{17}O$, and [^{17}O]CABP experiments, the isotopically enriched sample was matched to two other samples prepared with ^{16}O-containing components. This procedure allowed the verification that ^{16}O-enriched samples produced identical ESR spectra and validated the conclusion that spectral perturbations observed with ^{17}O-containing samples were due to the superhyperfine coupling of ^{17}O to Mn^{2+}.

ESR measurements of 9 GHz were made by using a Varian E-109 spectrometer equipped with 100-KHz field modulation. Temperature was maintained at 5 °C by blowing cooled nitrogen gas through a quartz variable-temperature Dewar insert and was measured by using a copper–constantan thermocouple in association with a Fluke 2100A digital thermometer. Measurements at 35 GHz were performed by using a Varian E-9 spectrometer equipped with an E-110 microwave bridge. Frozen samples were employed; temperature was maintained at −170 °C as described above. Field modulation was the same as indicated for the 9-GHz measurements. Care was taken to optimize the instruments so that base-line drift was not appreciable under the conditions used in recording the spectra.

The longitudinal relaxation rates ($1/T_1$) of water protons were measured at 24.3 MHz by using a SEIMCO pulsed NMR spectrometer operating at 22 °C. Measurements were made by the null-point method described by Mildvan & Engle (1972).

Results

ESR Measurements at 9 GHz of Mn^{2+} in the R. rubrum RuBP Carboxylase Quaternary Complex. Christeller (1981) has established the ability of Mn^{2+} to support both the carboxylase and oxygenase reactions catalyzed by *R. rubrum* RuBP carboxylase. Recently, it has been demonstrated that the dimeric *R. rubrum* enzyme will form a quaternary complex containing stoichiometric amounts of enzyme protomer, activator CO_2, CABP, and activator cation (Miziorko et al., 1982). The *R. rubrum* enzyme quaternary complex is similar to the model complex formed by using the spinach enzyme in that the enzyme-bound components are nonexchangeable under nondenaturing conditions.[2] Preparation of the quaternary complex using Mn^{2+} as the divalent cation and isolation of the stable complex free of unbound components permitted ESR observation of the stoichiometrically bound cation (Table I). At 9 GHz, solutions of the Mn^{2+}-containing *R. rubrum* quaternary complex produced the spectrum shown in Figure 1 (top). As observed in similar samples produced

[2] The *R. rubrum* enzyme quaternary complex is not as stable as the spinach enzyme complex (Donnelly et al., 1983). However, no exchange of bound components occurs at 4 °C in the few hours required to isolate samples for ESR studies.

Table I: Stoichiometry of Mn^{2+} Binding in the Sephadex G-75 Isolated *R. rubrum* RuBP Carboxylase–CO_2–Mn^{2+}–CABP Model Complex[a]

expt	Mn^{2+}/ enzyme site	CO_2/Mn^{2+}
1	1.22	0.99
2	1.17	1.03
3	1.21	1.00

[a] Enzyme was activated with 20 mM $KH^{14}CO_3$ (930 dpm/nmol) and 2.0 mM $MnCl_2$ prior to inactivation upon incubation (1 h at 30 °C) with 2 mM CABP. After the quaternary complexes were freed of unbound components by Sephadex G-75 chromatography (1.5 × 40 cm column; 50 mM Tris-HCl, pH 7.8), aliquots were subjected to analyses for $^{14}CO_2$ and Mn^{2+} content, and the remainder was used for ESR measurements.

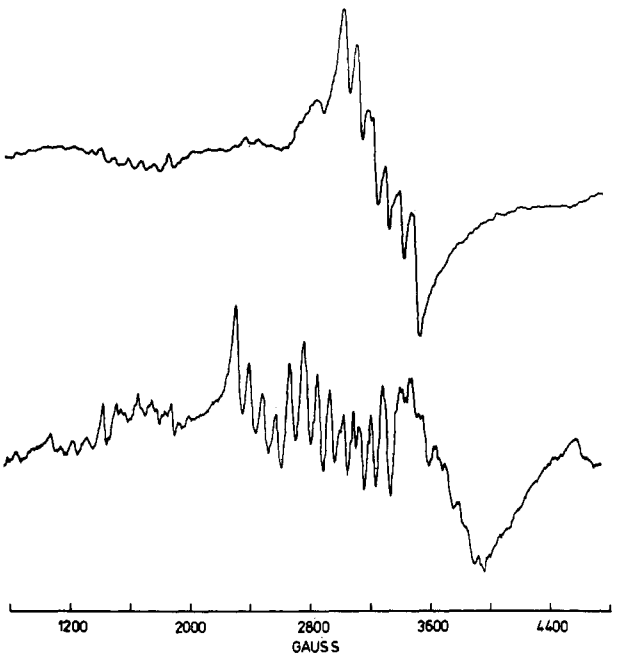

FIGURE 1: 9-GHz ESR spectra of Mn^{2+} bound to dimeric *R. rubrum* RuBP carboxylase. A solution of enzyme–CO_2–Mn^{2+}–CABP was prepared as described under Methods and isolated free of unbound components by Sephadex G-75 chromatography. The sample was concentrated to 1.3 mM prior to spectral measurements, which were made by using a 6.3-G modulation amplitude. The bottom spectrum represents a comparable sample prepared by using the octameric spinach leaf enzyme, which contains one large (56 000-dalton) peptide and one small (14 000-dalton) peptide per protomer. Spectra were measured on solutions of the samples (5 °C).

by using spinach RuBP carboxylase (Miziorko & Sealy, 1980), Mn^{2+} bound in the quaternary complex must experience a noncubic ligand field in order to account for the ESR signal. There are some similarities in the 9-GHz spectra measured by using dimeric *R. rubrum* (Figure 1, top) and octameric spinach (Figure 1, bottom) enzyme samples. For example, both samples produce spectra with substantial fine structure centered around $g = 4.3$. However, the *R. rubrum* quaternary complex samples generate little resolved signal intensity in the $g = 2.5$ region compared to the clearly observable signals downfield. In contrast, Mn^{2+} in the spinach enzyme quaternary complex gives rise to well-resolved peaks in the $g = 2.5$ region which are much more intense than the downfield peaks and are, in fact, comparable in intensity to the central fine structure transition, where most of the signal intensity from Mn^{2+} bound tightly to other proteins has typically been observed. Such differences indicate that Mn^{2+} experiences different zero-field splitting in the two complexes; differences in liganding could account for this.

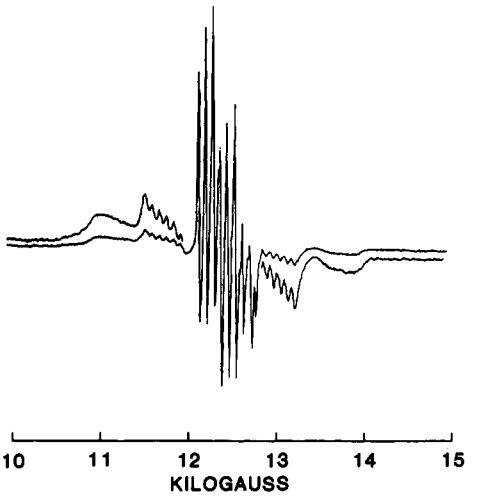

FIGURE 2: 35-GHz ESR spectrum of Mn^{2+} bound to spinach leaf RuBP carboxylase. A solution (1.7 mM enzyme sites) of the isolated enzyme–CO_2–Mn^{2+}–CABP complex was frozen in a 1-mm quartz capillary for examination by ESR. Measurements were performed at −170 °C, by using a 2.5-G modulation amplitude. Traces above and below the complete spectrum were recorded at a 3-fold increase in instrument gain.

Measurements at 35 GHz of Mn^{2+} in the Spinach Leaf RuBP Carboxylase Quaternary Complex: Resolution of Fine Structure Transitions. High-frequency (35 GHz) ESR measurements have been recommended for the investigation of enzyme-bound Mn^{2+} (Reed & Ray, 1971; Palmer, 1980) when a broad spectrum with solid-state characteristics is observed at 9 GHz. The 35-GHz spectrum is typically narrower than that observed at 9 GHz, facilitating computer modeling (Markham et al., 1979) or detection of small perturbations of the spectral shape. Initial 35-GHz measurements on Mn^{2+} model complexes prepared by using spinach enzyme and performed on samples in the solution state (Miziorko & Sealy, 1980) produced relatively uninformative spectra. Comparison of 9-GHz spectra generated from plant and bacterial RuBP carboxylase model complexes (Figure 1) suggested that more information should be available from the 35-GHz spectrum of Mn^{2+} in the plant enzyme complex and prompted reinvestigation of such samples in the frozen state. In contrast to 9-GHz spectra, which are not improved by measurements at low temperatures, the 35-GHz spectrum of Mn^{2+} in the frozen spinach leaf enzyme quaternary complex is quite informative. A powder spectrum is observed, indicative of noncubic symmetry and showing the expected orientation dependence of the fine structure transitions. Predicted fine structure transitions are observable between 11 and 14 kG (Figure 2). Hyperfine splitting is reasonably well resolved except for the peaks due to the $-5/2 \leftrightarrow -3/2$ and $+3/2 \leftrightarrow +5/2$ transitions, which appear on the wings of the spectrum. Nonetheless, these broader features are easily detectable, especially at increased gain (Figure 2, above and below complete spectrum). The central fine structure transition is split, reflecting a high value for the zero-field splitting (Reed & Ray, 1971). From the positions of the observed fine structure transitions at 35 GHz, a value for the zero-field splitting parameter ($D \geq 450$ G) can be estimated, by using the approach of Reed & Ray (1971).

Although the −170 °C spectrum generated from Mn^{2+} bound to the octameric plant enzyme represents a substantial improvement in resolution compared to the spectrum measured in the solution state, the features are too broad to readily permit detection of superhyperfine perturbations. In view of the simplified spectrum obtained with the dimeric bacterial

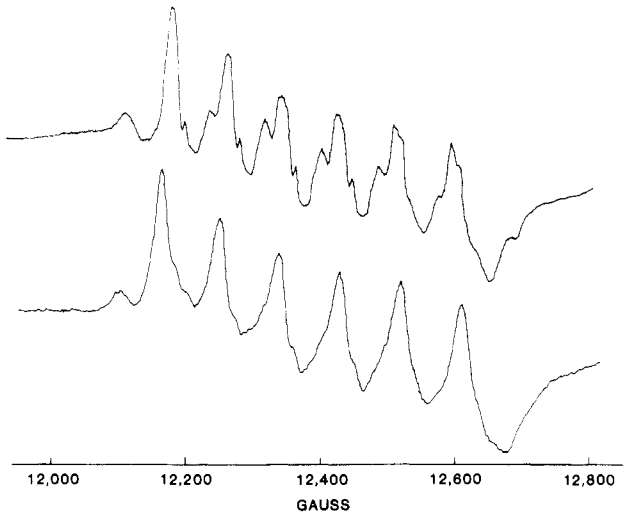

FIGURE 3: 35-GHz ESR spectra of Mn^{2+} bound to *R. rubrum* RuBP carboxylase. A solution (0.86 mM enzyme sites) of the isolated enzyme–CO_2–Mn^{2+}–CABP complex, prepared as described under Methods, was frozen in a 1-mm quartz capillary and maintained at −170 °C for spectral measurements. The bottom spectrum was measured similarly, by using a sample of the quaternary complex prepared in $H_2{}^{17}O$ (42% enrichment). The sample contains limiting Mn^{2+} concentration (0.86 mM vs. 1.03 mM enzyme sites) in order to assure complete incorporation of cation into the quaternary enzyme–CO_2–Mn^{2+}–CABP complex. A modulation amplitude of 2.5 G was used in recording both spectra.

enzyme at 9 GHz, it seemed appropriate to investigate these samples at high field and under conditions which improved resolution for Mn^{2+} bound to the octameric plant enzyme.

Measurements at 35 GHz of Mn^{2+} in the R. rubrum RuBP Carboxylase Quaternary Complex: Detection of Superhyperfine Coupling between Mn^{2+} and $H_2{}^{17}O$. In contrast to the high-field ESR spectrum of Mn^{2+} bound to octameric spinach enzyme, the spectrum due to Mn^{2+} bound to dimeric *R. rubrum* enzyme essentially shows only the central fine structure transition. Thus, the spectral intensity is centered about $g = 2$ (Figure 3, top), with the sextet resulting from hyperfine splitting due to the ^{55}Mn nucleus flanked by broad signals of lower intensity. By comparison with 9- and 35-GHz spectra of other Mn^{2+} proteins (Reed & Ray, 1971), the zero-field splitting is small ($D < 150$ G). Partially resolved features, attributable to second-order fine structure features on the powder pattern for the central fine structure transition, are observed on either shoulder of the major peaks of the sextet. Inflection points due to these features are separated by less than 10 G, and thus, on the basis of the criteria discussed by Reed & Leyh (1980), it seemed likely that these samples would be suitable for investigation of possible superhyperfine coupling of ^{17}O to Mn^{2+}.

^{17}O superhyperfine coupling to Mn^{2+} has been detected in ESR studies of several proteins (Reed & Leyh, 1980; Eccleston et al., 1981; Webb et al., 1982). The interaction between ^{17}O and Mn^{2+} may cause a visible broadening of the ESR spectrum (compared to a matched sample prepared by using ^{16}O) and obscure narrow spectral features. However, in some cases, the Mn^{2+} spectrum exhibits relatively broad spectral features even when measured in ^{16}O-containing samples and the ^{17}O effect is more difficult to detect, being manifested as a diminution in peak amplitude (Reed & Leyh, 1980). Difference spectra must be carefully constructed in order to convincingly demonstrate these less obvious perturbations, and solution studies are preferred in order to avoid any changes in peak amplitude which could arise from inhomogeneities in a frozen sample. Since the RuBP carboxylase samples produce useful spectra only in the frozen state, this investigation of ^{17}O effects relied on the detection of clearly broadened lines. The ESR spectrum of a Mn^{2+}-containing quaternary complex prepared in $H_2{}^{17}O$ (Figure 3, bottom) is clearly broadened in comparison with the spectrum of a comparable sample in $H_2{}^{16}O$. Virtually no trace of the partially resolved features observable in the ^{16}O sample (Figure 3, top) remains, arguing for the direct inner-sphere coordination of $H_2{}^{17}O$ to enzyme-bound Mn^{2+} in the quaternary complex.

Because of the marked stability of binding of activator CO_2 in the quaternary complex, it is possible to extend the ^{17}O studies and investigate Mn^{2+}–activator CO_2 interactions. If CO_2 is equilibrated in $H_2{}^{17}O$ (trace amounts of carbonic anhydrase will assure equilibration), $C^{17}O_2$ can be generated for use in forming activated enzyme. An ^{17}O-enriched carbamate is formed, and this species must remain ^{17}O enriched provided it does not dissociate to regenerate free CO_2, an event which is precluded upon addition of CABP to form the quaternary complex (Miziorko et al., 1982; Miziorko, 1979). In order to detect a Mn^{2+}–activator $C^{17}O_2$ interaction, it is necessary that the $H_2{}^{17}O$ used in forming the original sample be exchanged for unenriched H_2O or the large observed $H_2{}^{17}O$ effect would obscure any potential spectral broadening due to activator $C^{17}O_2$. Matched samples containing enzyme and Mn^{2+} were prepared in $H_2{}^{17}O$ with $^{17}CO_2$ or in $H_2{}^{16}O$ with $C^{16}O_2$. CABP was added to form exchange-inert complexes which were subjected to G-75 chromatography (buffer prepared in $H_2{}^{16}O$) to remove unbound components and allow $H_2{}^{17}O$–$H_2{}^{16}O$ exchange in the case of the ^{17}O-enriched sample. An additional sample, prepared by using $C^{17}O_2$ and $H_2{}^{17}O$, was not subjected to chromatography and served as a positive control, producing the bottom spectrum shown in Figure 3, due to $H_2{}^{17}O$ effects. ESR measurements on the Sephadex G-75 isolated $C^{17}O_2$-containing sample produced a spectrum which was undistinguishable from the spectra measured by using the Sephadex G-75 isolated $C^{16}O_2$-containing quaternary complexes. This indicates that the water molecules which ligand to Mn^{2+} do, in fact, exchange from the quaternary complex more rapidly than activator CO_2, CABP, or cation. Moreover, the failure to detect substantial superhyperfine coupling between Mn^{2+} and activator $C^{17}O_2$ means that direct evidence for inner-sphere coordination of activator CO_2 to cation remains to be reported.

Proton Relaxation Rate Studies of the RuBP Carboxylase Quaternary Complex. Previous studies on the spinach leaf enzyme (Miziorko & Mildvan, 1974) indicated that addition of a mixture of carboxypentitol bisphosphates to a ternary enzyme–CO_2–Mn^{2+} complex markedly reduced the enhancement of water proton relaxation due to enzyme-bound cation. Those data could not be unambiguously interpreted at that time, and in view of the $H_2{}^{17}O$ effects on the ESR spectrum of Mn^{2+} bound to dimeric *R. rubrum* RuBP carboxylase, it seemed that additional water relaxation experiments would yield useful data. Mn^{2+} bound to CO_2-activated *R. rubrum* carboxylase has an enhanced ability to relax water protons compared to Mn^{2+} free in aqueous solution (Figure 4). Upon titration of the ternary complex with purified CABP, enhancement of the proton relaxation rate decreases until, at concentrations of CABP approaching that of bound Mn^{2+} (indicated by an arrow on Figure 4), all effects of the cation are abolished. CABP rapidly exerts its effect on the dimeric *R. rubrum* enzyme complex; no lag is observed in reaching the final relaxation rate ($1/T_1$) after each addition of CABP. In contrast, titration of ternary spinach enzyme complexes with CABP resulted in slowly decreasing relaxation

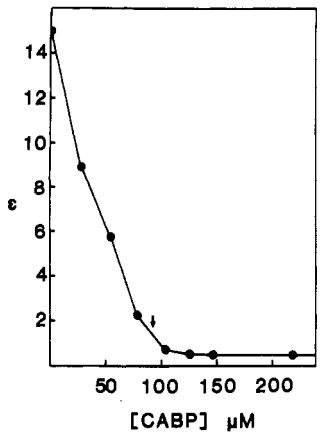

FIGURE 4: Titration of the ternary *R. rubrum* RuBP carboxylase–CO_2–Mn^{2+} complex with carboxyarabinitol bisphosphate (CABP). The enhancement of the water proton relaxation rate (ϵ) vs. the total concentration of CABP is depicted. The ternary complex contained 463 μM enzyme sites, 91 μM $MnCl_2$, and 30 mM $KHCO_3$ in 50 mM Tris-HCl, pH 7.8. The arrow indicates the point in the titration when added CABP equals the concentration of enzyme-bound cation. Temperature was 22 °C.

rates. Approximately 2–3 min elapsed after CABP addition before a stable relaxation rate could be measured. These observations suggest that the octameric plant enzyme experiences a conformational change upon tight CABP binding which is markedly different from any perturbation in conformation of the bacterial enzyme, which lacks small subunits.

Elimination of the ability of Mn^{2+} to relax solvent water protons upon formation of the quaternary complex could be accounted for by several mechanisms, including total displacement of bound water by CABP or occlusion of bound water so that rapid exchange with the bulk solvent water molecules does not occur. While solvent may be displaced upon CABP binding, ESR experiments in $H_2{}^{17}O$ (Figure 3, bottom) clearly indicate that some water remains in the inner coordination sphere of bound Mn^{2+}. Thus, the latter explanation, i.e., the existence of Mn^{2+}-coordinated water that exchanges slowly (on an NMR time scale) with the medium, remains an attractive possibility. While such water molecules can be considered "strictly" in the short NMR time frame, the data presented above indicated that, in the *R. rubrum* quaternary complex, these water molecules will completely exchange after 1–2 h at 4 °C and, therefore, are not occluded from the aqueous medium in any absolute sense.

Superhyperfine Coupling between Enzyme-Bound Mn^{2+} and ^{17}O-Enriched CABP. Formation of the *R. rubrum* RuBP carboxylase–activator CO_2–Mn^{2+}–CABP model complex with ^{17}O-enriched CABP permits investigation of Mn^{2+}–substrate analogue interactions. Matched G-75-isolated samples of the quaternary model complex prepared by using [^{16}O]CABP produced similar 35-GHz ESR spectra, depicted in Figure 5A,B. When a comparable sample, prepared by using CABP enriched with ^{17}O in the carboxyl oxygens, was investigated, the ESR spectrum shown in Figure 5C was observed. Consistent broadening across the entire spectrum is evident; the partially resolved features flanking the six major peaks are most dramatically affected. A matched sample was also prepared by using CABP enriched with ^{17}O in the alcoholic oxygen on C-2. After G-75 isolation in parallel with the samples described above, the ESR spectrum of this quaternary complex was measured and is shown in Figure 5D. Again, an obvious broadening of spectral features is observed. These manifestations of superhyperfine coupling between Mn^{2+} and ^{17}O argue for inner-sphere coordination between the cation

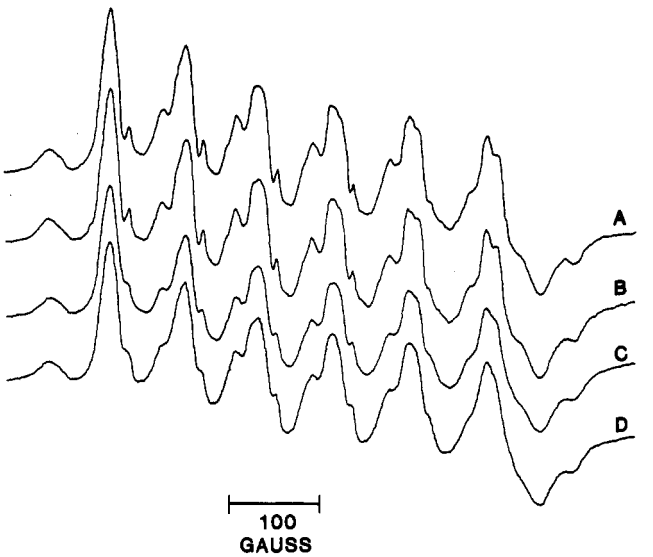

FIGURE 5: 35-GHz ESR spectra of Mn^{2+} bound to *R. rubrum* RuBP carboxylase quaternary complexes prepared by using ^{16}O- or ^{17}O-enriched carboxyarabinitol bisphosphate (CABP). Solutions (1.3 mM enzyme sites) of the Sephadex G-75 isolated quaternary enzyme–CO_2–Mn^{2+}–CABP complex, prepared as described under Methods, were frozen in 1-mm quartz capillaries and maintained at −170 °C for spectral measurements, which were made by using a 2.5-G modulation amplitude. Spectra A and B were generated from samples containing [^{16}O]CABP. Spectrum C was measured by using a sample prepared with CABP that was 30% enriched with ^{17}O in the carboxyl group oxygens. Spectrum D was measured by using a sample prepared with CABP that was 45% enriched with ^{17}O in the alcoholic oxygen on C-2.

and both the carboxyl group and the alcohol on C-2 of CABP in the quaternary complex. Thus, the data demonstrate proximity between activator cation and the substrate binding domain.

Discussion

Distortion of the coordination sphere of Mn^{2+} bound in the quaternary complex containing RuBP carboxylase, cation, activator CO_2, and CABP was first observed in studies on the spinach enzyme (Miziorko & Sealy, 1980), and a complex liganding arrangement was postulated to account for the ESR data. Such an argument is compatible with the extremely slow dissociation of components from the isolated complex (Miziorko, 1979; Miziorko & Sealy, 1980; Schloss & Lorimer, 1982; Pierce et al., 1980). Previously, in accounting for nonexchangeability of bound cation, we suggested that an explanation involving complex inner-sphere liganding of cation was more likely than the alternative hypothesis of occlusion of the cation binding domain from the bulk of the aqueous medium (Miziorko & Sealy, 1980). Recently, the results of several types of experiments have confirmed the original hypothesis. Direct liganding of cation to protein can be demonstrated under denaturing conditions using exchange-inert cations to form the initial quaternary complex (Miziorko et al., 1982). Inner-sphere liganding of Mn^{2+} to CABP is evident from the detection of superhyperfine coupling described in this report. Superhyperfine coupling between ^{17}O and Mn^{2+} may be ascribed to through-bond (scalar) and through-space (dipolar) interactions between nuclear and electronic spins. As discussed by Reed & Leyh (1980), the through-bond interaction is likely to make the largest contribution to any observed effect. Since the magnitude of the ^{17}O-dependent spectral splitting (Zetter et al., 1978) is small in comparison with the intrinsic spectral feature, in order for an ^{17}O nucleus to exert a measurable effect, it must be in the first coordination sphere

of Mn^{2+} (Reed & Leyh, 1980).

In ESR experiments using [^{17}O]CABP to show interaction with bound Mn^{2+}, a demonstration of proximity between the activator cation binding domain and the catalytic site has been achieved. Experiments involving exchange-inert cations failed to show stable direct binding of metal to CABP, but under the conditions employed, great stability of a metal–CABP adduct involving oxygen as the bridging atom was not expected (Miziorko et al., 1982). Thus, the data presented here are compatible with that earlier study. Proximity between activator cation and the catalytic site is also suggested by earlier ^{13}C NMR work (Miziorko & Mildvan, 1974). The rapidly exchanging species of CO_2 in mixtures of spinach enzyme–$H^{13}CO_3^-$–Mn^{2+} was shown to be bound close to the activator cation. In fact, a distance of 5.4 Å was calculated on the basis of the Solomon–Bloembergen theory, which is compatible with second-sphere liganding of CO_2 to metal. Several assumptions are made in applying the NMR theory for any distance calculation, and the correlation time for the observed interaction must be known (Mildvan & Cohn, 1970). However, any errors or approximations are reduced by the inverse sixth root dependence of the calculated distance on the measured relaxation effect. Thus, it remains possible that the Mn^{2+}–substrate CO_2 distance in enzyme–CO_2–Mn^{2+} mixtures is, in fact, longer than the distance between Mn^{2+} and the carboxyl group of CABP in the quaternary complex. It would be interesting to determine whether any superhyperfine coupling could be detected between a rapidly exchangeable $C^{17}O_2$ species and Mn^{2+} in an enzyme–CO_2–Mn^{2+} sample. The absence of such an effect would be compatible with the hypothesis that Mn^{2+} binds directly to a CO_2 species (occupying the substrate CO_2 site) only after binding of the sugar substrate.[3] However, the strong superhyperfine coupling between solvent $H_2{}^{17}O$ (with which $C^{17}O_2$ equilibrates) and bound Mn^{2+} would mask any spectral effect, making such an experiment impractical. Thus, other approaches will have to be employed in order to test this point.

Upon binding of CABP to enzyme–CO_2–M^{2+}, a marked conformational change occurs (Siegel & Lane, 1972). The slow kinetics of this event are reflected in the several minute delay before stable water proton relaxation rates are measured in titration experiments with the spinach enzyme. It is certainly possible that the group occupying the substrate CO_2 pocket moves closer to the cation as a result of this process. While this site is occupied by the carboxyl group of CABP in these model complex studies, in the actual transition state a carboxyl group of the six-carbon intermediate, 2-carboxy-3-ketoarabinitol bisphosphate, would occupy the substrate CO_2 site prior to product release (Miziorko & Lorimer, 1983). Formation of an *aci*-acid species of 3-phosphoglycerate has been postulated to occur uponcleavage of the six-carbon reaction intermediate (Pierce et al., 1980). Mn^{2+} coordinated directly to the negatively charge *aci*-acid would serve very efficiently as an electron sink, stabilizing such a species. Therefore, it appears that, on chemical grounds, as well as on the basis of the ESR data presented in this report, a direct role for the cation in the catalysis of RuBP carboxylation is likely.

The coordination of the cation to the alcoholic oxygen on C-2 of CABP also seems reasonable on chemical grounds. A stable five-membered ring is formed if Mn^{2+} binds oxygen atoms from the alcohol and carboxyl moieties of CABP. Moreover, deprotonation at C-3 of RuBP requires the presence

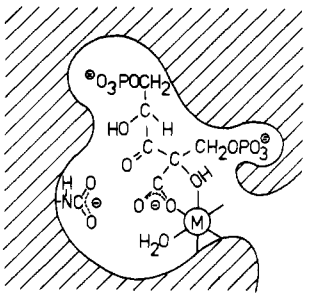

FIGURE 6: Schematic view of activators and substrates in the transition state catalyzed by ribulosebisphosphate carboxylase. Activator CO_2 is depicted as a carbamate, as established by Lorimer & Miziorko (1980) for the spinach leaf enzyme. Although the six-carbon intermediate 3-keto-2-carboxyarabinitol bisphosphate is depicted, data establishing coordination of cation to protein, substrates, and water were generated in experiments using the transition-state analogue carboxyarabinitol bisphosphate.

of cation (Saver & Knowles, 1982). This requirement had been predicted (Miziorko & Mildvan, 1974) on the basis of the cation's ability to function as an effective electron sink and to stabilize the enediol produced subsequent to deprotonation at C-3 by coordination to alcoholic oxygens at C-2 or C-3.

A model which accommodates the available structural information concerning the active site of RuBP carboxylase is presented in Figure 6. Much of the available information is derived from studies on the CABP-containing quaternary complex, although the schematic depicts 2-carboxy-3-ketoarabinitol bisphosphate, the six-carbon intermediate that is approximated by the transition-state analogue (Pierce et al., 1980; Schloss & Lorimer, 1982). While the number of water molecules bound to M^{2+} during turnover is still a matter of speculation, the magnitude of the ^{17}O broadening due to solvent is compatible with the assignment of multiple water ligands. Effects of similar magnitude were observed by Reed & Leyh (1980) in experiments on creatine kinase, which contains three water molecules in the enzyme complex that was studied. Additional physical experiments on RuBP carboxylase will be required before a more precise assignment can be made. Similarly, the number of enzyme-donated ligands has not yet been precisely determined, although multidentate liganding seems likely (Cleland & Mildvan, 1979) on the basis of the stability of Co^{3+} binding in denatured samples of the quaternary complex (Miziorko et al., 1982). The model represents activator CO_2 as the carbamate species which has been demonstrated to form on the spinach enzyme upon binding of CO_2 to the ϵ-amino group of a lysine (Miziorko & Lorimer, 1980). Direct coordination of the carbamate to cation is *not* depicted to underscore the fact that no *direct* evidence for a cation–activator CO_2 interaction has yet been obtained (Miziorko et al., 1982; Miziorko & Lorimer, 1983) despite the fact that the carbamate must be stabilized by a positively charge group (M^{2+} would be an excellent candidate in order to explain the nonexchangeability of activator CO_2 in the quaternary model complex). Thus, no complete assignment of metal ligands is currently possible, and the issue of a metal-stabilized carbamate remains to be resolved by future studies on M^{2+}–activator CO_2 liganding or by unambiguous quantitation of solvent- and protein-donated ligands, which could rule out a cation–activator CO_2 interaction.

The model shown in Figure 6 relies heavily on data derived from ESR studies which have required the use of *R. rubrum* RuBP carboxylase. The question of whether it is prudent to extrapolate from data derived by using the dimeric bacterial enzyme to a structure pertinent to the octameric plant enzyme (or vice versa) might well arise. The differences in the ESR

[3] Schloss (1983) has generated kinetic data which suggest that both activator and substrate CO_2 molecules can bind to enzyme in the absence of RuBP.

spectra of Mn^{2+} bound to these enzymes suggest possible changes in liganding of the cation. In addition, the quaternary complex formed by using the *R. rubrum* enzyme is somewhat less stable than the complex prepared by using spinach RuBP carboxylase (Donnelly et al., 1983). However, available information suggests that these enzymes are quite similar in many features which are important to the activation and catalytic events. Both enzymes undergo CO_2 activation (Lorimer et al., 1976; Christeller & Laing, 1978) and form exchange-inert quaternary complexes in the presence of the transition-state analogue (Miziorko et al., 1982). An active-site lysyl residue has been identified on both enzymes (Stringer & Hartman, 1978; Whitman & Tabita, 1978; Donnelly et al., 1983). Striking homologies in primary sequence are observed in those regions which have been implicated in substrate binding, despite the fact that the total amino acid compositions of the plant and bacterial enzymes are quite dissimilar (Hartman et al., 1982). Thus, it seems reasonable to make use of the scheme depicted in Figure 6 as a working model until future elucidation of any functionally important differences between plant and bacterial enzymes leads to a more refined description of the enzyme's active site.

Acknowledgments

Dr. Fred Hartman generously provided the *R. rubrum* RuBP carboxylase used in these experiments. We are grateful to Dr. Leon Saryan (West Allis Memorial Hospital, West Allis, WI) for performing atomic absorption measurements of Mn^{2+} in the model complexes, to Dr. Jim Cook and Frank Labe (University of Wisconsin–Milwaukee) for making possible the mass spectroscopic analyses of ^{17}O-enriched CABP, and to Drs. J. Pierce and G. Lorimer (E. I. du Pont de Nemours and Co., Inc., Wilmington, DE) for useful discussions on labeling strategies. The National Biomedical ESR Center (Milwaukee, WI) made available the instrumentation used in our ESR experiments. Scott Hill provided excellent technical assistance.

Registry No. CABP, 27442-42-8; RuBP carboxylase, 9027-23-0; Mn, 7439-96-5.

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