Friedman, P. A., & Shia, M. (1976) *Biochem. Biophys. Res. Commun. 70*, 647–654.
Gram, T. E. (1974) *Methods Enzymol. 31*, 225–237.
Grant, G. A., & Suttie, J. W. (1976) *Biochemistry 15*, 5387–5393.
Helgeland, L. (1977) *Biochim. Biophys. Acta 499*, 181–193.
Ito, A., & Sato, R. (1969) *J. Cell Biol. 40*, 179–189.
Kao, W. W.-Y., & Lee, K.-L. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, Abstr. No. 1420.
Kasper, C. B. (1974) *Methods Enzymol. 31*, 279–292.
Knauer, T. E., Siegfried, C. M., & Matschiner, J. T. (1976) *J. Nutr. 106*, 1747–1756.
Kreibich, G., Hubbard, A. L., & Sabatini, D. D. (1974) *J. Cell Biol. 60*, 616–627.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Mack, D. O., Suen, E. T., Girardot, J.-M., Miller, J. A., Delaney, R., & Johnson, B. C. (1976) *J. Biol. Chem. 251*, 3269–3276.
Morre, D. J. (1971) *Methods Enzymol. 22*, 130–148.
Nilsson, R., Peterson, E., & Dallner, G. (1973) *J. Cell Biol. 56*, 762–776.
Nyquist, S. E., Matschiner, J. T., & Morre, D. J. (1971) *Biochim. Biophys. Acta 244*, 645–649.
Olson, B. R., & Prockop, D. J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2033–2037.
Sadowski, J. A., Schnoes, H. K., & Suttie, J. W. (1977) *Biochemistry 16*, 3856–3863.
Shah, D. V., & Suttie, J. W. (1978) *Arch. Biochem. Biophys. 191*, 571–577.
Siegfried, C. M. (1978) *Biochem. Biophys. Res. Commun. 83*, 1488–1495.
Sottocasa, G. L., Kuylenstierna, B., Ernster, L., & Bergstrand, A. (1967) *Methods Enzymol. 10*, 448–463.
Spelsberg, T. C., Knowler, J. T., & Moses, H. R. (1974) *Methods Enzymol. 31*, 263–279.
Steck, T. L. (1972) in *Membrane Molecular Biology* (Fox, C. F., & Keith, A. D., Eds.) p 80, Sinauer, Stamford, CT.
Stenflo, J. (1978) *Adv. Enzymol. Relat. Areas Mol. Biol. 46*, 1–31.
Suttie, J. W. (1978) *Handb. Lipid Res. 2*, 211–277.
Suttie, J. W. (1979) *CRC Crit. Rev. Biochem.* (in press).
Suttie, J. W., & Jackson, C. M. (1977) *Physiol. Rev. 57*, 1–70.
Suttie, J. W., Hageman, J. M., Lehrman, S. R., & Rich, D. H. (1976) *J. Biol. Chem. 251*, 5827–5830.
Suttie, J. W., Larson, A. E., Canfield, L. M., & Carlisle, T. L. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 2605–2609.
Suttie, J. W., Lehrman, S. R., Geweke, L. O., Hageman, J. M., & Rich, D. H. (1979) *Biochem. Biophys. Res. Commun. 86*, 500–507.
Thierry, M. J., & Suttie, J. W. (1971) *Arch. Biochem. Biophys. 147*, 430–435.
Wallin, R., & Prydz, H. (1979) *Thromb. Haemostasis 41*, 529–536.
Wallin, R., Gebhardt, O., & Prydz, H. (1978) *Biochem. J. 169*, 95–101.
Whitlon, D. S., Sadowski, J. A., & Suttie, J. W. (1978) *Biochemistry 17*, 1371–1377.
Willingham, A. K., & Matschiner, J. T. (1974) *Biochem. J. 140*, 435–441.
Zimmerman, A., & Matschiner, J. T. (1974) *Biochem. Pharmacol. 23*, 1033–1040.

Characterization of the Ribulosebisphosphate Carboxylase–Carbon Dioxide–Divalent Cation–Carboxypentitol Bisphosphate Complex†

Henry M. Miziorko* and Roger C. Sealy

ABSTRACT: Ribulosebisphosphate carboxylase forms a stable quaternary complex with CO_2, divalent cation, and carboxypentitol bisphosphate. Incorporation of nonexchangeable CO_2 into the complex requires the presence of a divalent cation. Mg^{2+}, Mn^{2+}, or Co^{2+} supports stoichiometric binding of CO_2 activator. When the quaternary complex is formed in the presence of saturating CO_2, stoichiometric amounts of cation are bound in a nonexchangeable fashion. Incorporation of Mn^{2+} into an enzyme–CO_2–Mn^{2+}–carboxypentitol bisphosphate complex permitted investigation of cation environment by electron spin resonance (ESR) techniques. Measurements at 9 and 35 GHz suggest rhombic distortion of the coordination sphere of bound Mn^{2+}. A complex inner sphere liganding of the cation bound in the quaternary complex would account for both the ESR spectra and the marked stability of the complex with respect to cation exchange.

The primary reaction in photosynthetic carbon fixation is catalyzed by ribulosebisphosphate carboxylase, an enzyme which requires a divalent metal cation (M^{2+}) for M^{2+} (Weissbach et al., 1956; Siegel et al., 1972). Kinetic (Lorimer et al., 1976) and physical (Miziorko & Mildvan, 1974) evidence suggests that there is an ordered addition of CO_2 and M^{2+} to the purified enzyme. Upon formation of this ternary complex, the enzyme becomes catalytically competent and the dissociation constant of the cation approaches the activator constant determined kinetically. Observation of an activation process prompted speculation that the enzyme binds an activator CO_2 molecule that is distinct from the CO_2 which is ultimately fixed in the carboxylation reaction. Kinetic turnover experiments (Lorimer, 1979) and the demonstration of simultaneous stoichiometric binding of CO_2 and the transition-state analogue carboxypentitol bisphosphate (CPBP)[1] to

† From the Departments of Biochemistry and Radiology, The Medical College of Wisconsin, Milwaukee, Wisconsin 53226. *Received November 14, 1979.* Supported in part by U.S. Department of Agriculture Grant 5901-0410-0339-0, National Science Foundation Grant PCM-7911474, and National Institutes of Health Grant 3 P41 RR01008-03S1.
* Correspondence should be addressed to this author. He is a U.S. Public Health Service Research Career Development Awardee (AM 00645).

0006-2960/80/0419-1167$01.00/0

the enzyme (Miziorko, 1979) provide support for the two CO_2 site hypothesis.

The suggestion of a dual role for CO_2 in enzyme activation as well as in catalysis leads to a question of whether M^{2+} also has a dual function. The role of cation in activation seems to be established, but its function in catalysis has been questioned (Laing & Christeller, 1976). The efficiency of the enzyme in catalyzing carboxylation and the related oxygenation process appears to depend on which cation is supplied to the enzyme (Wildner & Henkel, 1978, 1979; Robison et al., 1979). It is unclear whether these observations can be accounted for by postulating differential enzyme activation or whether differential efficiency of cations in supporting catalysis should be considered. The question of the stoichiometry of M^{2+} binding during enzyme turnover also seems worthy of closer scrutiny in light of recent work on the activation process.

This report describes experiments employing the model complex formed by E–CO_2–M^{2+}–CPBP.[2] Previous work (Siegel & Lane, 1972; Miziorko, 1979) suggests that the complex provides a useful system for studying enzyme activation and catalysis. The data presented here add to an understanding of the model, demonstrate more clearly the participation of cation in the stabilization of the complex, and suggest an unusual environment for cation immobilized in the complex. A preliminary account of these studied has appeared (Miziorko, 1978).

Experimental Section

Materials. RuBP carboxylase was prepared from spinach leaves as described by Paulsen & Lane (1966) and assayed by either standard radioisotopic (Lorimer et al., 1977) or spectrophotometric (Andrews et al., 1973) procedures. Protein concentration was determined spectrophotometrically by using the extinction coeffecient previously reported (Paulsen & Lane, 1966).

Ribulose bisphosphate was purchased from Sigma Chemical Co. Carboxypentitol bisphosphate was prepared as described by Siegel & Lane (1973) and purified by chromatography on DEAE-cellulose. $NaH^{14}CO_3$ and $^{57}CoCl_2$ were purchased from New England Nuclear. Chelex 100 resin was obtained from Bio-Rad Laboratories. Sephadex G-25 and Sephadex G-75 were purchased from Pharmacia. 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) with 0.5 mM DTT. Reagents and buffers were Chelex-treated before use. Enzyme activation was accomplished by addition of $KHCO_3$ to approximately 20 mM and divalent cation to the concentration indicated in the text. After incubation of the mixture for 15 min at 30 °C, a fivefold excess (with respect to enzyme sites) of CPBP was added and allowed to react for 1 h at 30 °C. In experiments where stoichiometric amounts of CO_2 were irreversibly bound in the enzyme complex, typical inhibition of enzyme activity was greater than 95%. Separation of enzyme complex from unbound components in the incubation mix was accomplished by gel filtration using a 1.5 × 40 cm Sephadex G-75 column. Isolated samples were concentrated, if necessary, by use of a collodion sack apparatus (Schleicher & Schuell). Measurement of ^{14}C radioactivity was performed by bringing an aliquot of sample to 0.3-mL aqueous volume and mixing with 3 mL of a toluene–Triton base scintillation fluid prior to scintillation counting. γ emission due to $^{57}Co^{2+}$ was measured by using a Beckman γ Mate 100 counter. Atomic absorption analysis was performed on a Varian AA-5 equipped with a carbon rod furnace. The 9-GHz ESR measurements were performed on a Varian E-109 spectrometer equipped with 100-kHz field modulation. Samples (60 μL) were contained within a quartz variable temperature flat cell (James F. Scanlon Co., Model S-808). The temperature of the sample was maintained at 0.5 °C by blowing cold nitrogen gas through a quartz variable temperature Dewar insert and was measured by using a thermocouple in association with a Fluke 2100A digital thermometer. The 35-GHz measurements were carried out on ~2-μL samples in capillaries. A Varian E-9 spectrometer equipped with an E-110 microwave bridge was used for these measurements. Field modulation and temperature control and measurement were the same as for the 9-GHz measurements. Modulation amplitude was selected to maximize the signal without artifically broadening spectral lines. Base line drift was not appreciable under the conditions used in recording the spectra shown here. Microwave power was selected such that saturation of the ESR resonances was not observed in either the 9- or 35-GHz experiments.

Table I: Stoichiometry of CO_2 Trapping in the RuBP Carboxylase–CO_2–M^{2+}–Carboxypentitol Bisphosphate Complex[a]

activating cation	CO_2/enzyme site in Sephadex G-75 purified complex	CO_2/site after exchange vs. 50 mM $KHCO_3$
Mg^{2+}	0.91	0.80
Mn^{2+}	1.06	0.97
Co^{2+}	0.91	0.79
Cu^{2+}	0.14	0.08
none	0.44	0.10

[a] Enzyme (5 mg) is incubated with 20 mM $KH^{14}CO_3$ (1000 dpm/nmol) and metal ion (Mg^{2+} = 10 mM; Mn^{2+}, Co^{2+}, and Cu^{2+} = 2 mM). In experiments without added cation, either no addition or addition of 1 mM EDTA was made. After 15 min at 30 °C, a fivefold excess of carboxypentitol bisphosphate is added. One hour after CPBP addition, the reaction mixture is cooled to 4 °C and loaded into a Sephadex G-75 column (40 × 1.5 cm). Upon elution of the column, enzyme-bound $^{14}CO_2$ is well separated from unbound CO_2 and M^{2+}, permitting calculation of the stoichiometry of binding. The isolated enzyme complex (typically 30–50 μM in enzyme sites and bound $^{14}CO_2$) is brought to 50 mM in unlabeled $KHCO_3$, incubated for 1 h at 30 °C, and then rechromatographed on Sephadex G-75. ^{14}C radioactivity is measured across the resulting protein peak, and the CO_2/enzyme site is calculated. Experiments using 0.2 mM Co^{2+} in the original incubation medium produced results similar to those observed when 2.0 mM Co^{2+} was used.

Results

Investigations by several laboratories have suggested that CO_2 fixation by RuBP carboxylase is metal dependent and that the requirement is satisfied, with variable efficiency, by Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, and Fe^{2+} (Weissbach et al., 1956; Paulsen & Lane, 1966; Wildner & Henkel, 1978, 1979). With the recent demonstration of cation involvement in activation, a question arose concerning the efficiency of various cations in supporting activation. The approach employed here to

[1] Abbreviations used: RuBP, ribulose bisphosphate; CPBP, carboxypentitol bisphosphate; M^{2+}, divalent metal cation; $NaDodSO_4$, sodium dodecyl sulfate; ESR, electron spin resonance.

[2] The carboxypentitol bisphosphate is prepared as reported by Siegel & Lane (1973) and consists of a mixture of carboxyarabinitol and carboxyribitol bisphosphates. Recent work of Pierce et al. (1979) suggests that carboxyarabinitol bisphosphate is the analogue which forms the functionally irreversible complex. In the experiments reported here, enzyme is inhibited with excess CPBP and incubated for a sufficient period (1 h) so that irreversible inactivation by the tight binding analogue will result, accounting for the properties of the complex described in this paper. However, since the mixture has not been chromatographically resolved, the term carboxypentitol bisphosphate will be used.

Table II: Stoichiometry of Metal Binding in the Isolated RuBP Carboxylase-CO_2-M^{2+}-CPBP Complex[a]

activating cation	[M^{2+}] in incubation mix (mM)	M^{2+}/enzyme site	detection technique
Mn^{2+}	2.0	1.2 ± 0.2	atomic absorption
Co^{2+}	0.2	1.1 ± 0.2	$^{57}Co/\gamma$ emission
Co^{2+}	2.5	>3.5	$^{57}Co/\gamma$ emission; atomic absorption

[a] Enzyme (7 mg) is preincubated with 20 mM $KHCO_3$ and cation for 15 min at 30 °C prior to inhibition with a fivefold excess of CPBP. One hour after CPBP addition, the enzyme complex is isolated as described in Table I. Concentration of bound cation in the isolated E-CO_2-M^{2+}-CPBP complex was measured by atomic absorption or by γ counting in experiments which employed $^{57}CoCl_2$ (sp act. 700 cpm/nmol).

address that question involves determination of the amount of activator CO_2[3] locked irreversibly into the E-CO_2-M^{2+}-CPBP complex. Enzyme is preincubated with $^{14}CO_2$ and cation and then incubated with an excess (CPBP/site ≥ 5) of transition-state analogue. Upon completion of inhibition, the enzyme-bound material is separated from unbound material by chromatography on Sephadex G-75. Measurement of ^{14}C radioactivity and protein concentration permits calculation of the stoichiometry of CO_2 binding. Table I shows that Mg^{2+}, Mn^{2+}, and Co^{2+} support stoichiometric binding of CO_2. In the absence of a cation or in the presence of Cu^{2+}, there is considerable diminution of CO_2 bound. Moreover, when the isolated enzyme complex (typical concentration is less than 50 μM) is allowed to exchange against 50 mM $KHCO_3$ prior to reisolation of enzyme by gel filtration, the nonexchangeable nature of stoichiometrically bound activator CO_2 is apparent only in the experiments with Mg^{2+}, Mn^{2+}, and Co^{2+}. Substoichiometric binding of an exchangeable CO_2, which is observed in the experiment performed in the absence of added cation, is compatible with the observation of bimodel inhibition of enzyme activity by CPBP. When incubation of enzyme, Mg^{2+}, and CPBP was performed at 4 °C, the inhibition of enzyme activity was reversible and substoichiometric binding of CPBP was observed (Siegel & Lane, 1972). At 30 °C, an irreversible inhibition was described. This finding was explained by invoking a conformational change between the two forms of CPBP-inhibited enzyme. The hypothesis has been substantiated by the recent report of Pierce et al. (1979), which shows that a two-step process provides a good working model for explaining CPBP's inhibitory properties. The observation (Table I) that cation is required for trapping of nonexchangeable CO_2 suggests that, if some conformational change results in an irreversible stabilization of the CPBP-containing complex, divalent cation is involved in the stabilization.

The assignment of roles in activation and catalysis to the divalent cation would be less controversial if the binding of two cations per active site was demonstrated. In experiments on the ternary enzyme-CO_2-Mn^{2+} complex, a stoichiometry of one cation per site was clearly established (Miziorko & Mildvan, 1974). In previous studies on CPBP inhibition (Wishnick et al., 1970), where the level of CO_2 activation was

[3] CO_2 is considered to be the substrate (Cooper et al., 1969) and activator (Lorimer et al., 1976) of the enzyme. Because CO_2 is in equilibrium with HCO_3^-, both species will be present in the experiments described here. The designations CO_2 and HCO_3^- are used interchangeably throughout this report, although at the pH values of the experiments HCO_3^- is the predominant species.

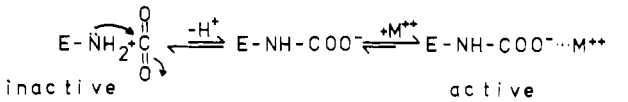

FIGURE 1: A possible explanation for CO_2 and cation involvement in activation of RuBP carboxylase.

Table III: Nonexchangeability of the Bound Cation in the RuBP Carboxylase-CO_2-M^{2+}-Carboxypentitol Bisphosphate Complex[a]

M^{2+} incorpd into E-CO_2-M^{2+}-CPBP	competing cation	% exchange	method of detection
Mn^{2+}	Mg^{2+}	4.3	ESR
$^{57}Co^{2+}$	$^{59}Co^{2+}$	3.1	γ emission

[a] Enzyme is preincubated with 20 mM $KHCO_3$ and 2 mM $MnCl_2$ or 0.2 mM $^{57}CoCl_2$ (700 cpm/nmol) for 15 min at 30 °C prior to the addition of CPBP (fivefold excess with respect to enzyme sites). After an additional 1-h incubation at 30 °C, E-CO_2-M^{2+}-CPBP was isolated by Sephadex G-75 chromatography. Samples were then brought to a 100-fold excess in competing cation. Isolated E-CO_2-Mn^{2+}-CPBP (1 mM; 70 μL) was allowed to exchange against 100 mM $MgCl_2$ for 16 h at 30 °C prior to estimate of exchange by observation of free and bound Mn^{2+} by ESR. $[Mn^{2+}]_{free}$ was determined by comparing the observed signal amplitude with that of Mn^{2+} standards run under identical conditions. Isolated E-CO_2-$^{57}Co^{2+}$-CPBP (6 μM; 4.4 mL) was brought to 0.6 mM in $^{59}CoCl_2$ and incubated for 2 h at 30 °C prior to reisolation of the complex by Sephadex G-75 chromatography. Stoichiometry of Co^{2+}/enzyme was invariant across the protein peak and was compared to the original stoichiometry in order to estimate exchange.

not controlled, the stoichiometry of CPBP/M^{2+} ranged from 1 for "native" enzyme to 2 for "copper-free" enzyme. It seemed appropriate to investigate metal binding to enzyme in the presence of CPBP under conditions where the CO_2 activation site was saturated; the results of these experiments are summarized in Table II. At cation concentrations which are adequate to allow irreversible stoichiometric binding of activator CO_2 in the isolated E-CO_2-M^{2+}-CPBP complex, only one cation per active site is bound. Thus, if cation has a role in both activation and catalysis, a single metal must have dual function. In experiments at elevated Co^{2+} concentration, while stoichiometric CO_2 is bound in the presence of CPBP, several Co^{2+}'s per active site are observed after gel filtration of the complex. However, in such a sample the stoichiometry of Co^{2+}/site is variable across the protein peak, unlike the constant value observed across the peak in the experiment at lower Co^{2+} levels. Thus, the additional Co^{2+} appears to be nonspecifically bound and may represent a class of weak sites created upon CPBP binding to enzyme. In the absence of CPBP, gel filtration of a ternary E-CO_2-$^{57}Co^{2+}$ mixture results in an enzyme species which is virtually Co^{2+} free (data not shown).

In view of the nonexchangeable nature of CO_2 binding in the E-CO_2-M^{2+}-CPBP complex, it seemed appropriate to test how irreversibly cation was bound in the complex. The ability of cation to activate the enzyme has been explained (Figure 1) by postulating that it stabilizes a carbamate formed between enzyme and activator CO_2 (Lorimer et al., 1976; O'Leary et al., 1979). Such a complex should be reversible, and, in fact, the ternary E-CO_2-$^{57}Co^{2+}$ complex described above readily dissociates upon gel filtration. However, in the presence of CPBP, activated enzyme binds metal more tenaciously (Table II). Cation exchangeability was tested by preparing isolated E-CO_2-M^{2+}-CPBP, mixing the complex with excess cation, and monitoring the cation originally bound. In experiments with isolated E-CO_2-Mn^{2+}-CPBP, Mg^{2+} (100-fold excess) was used as the competing cation (Table III). The tightly bound Mn^{2+} could be monitored by its characteristic ESR

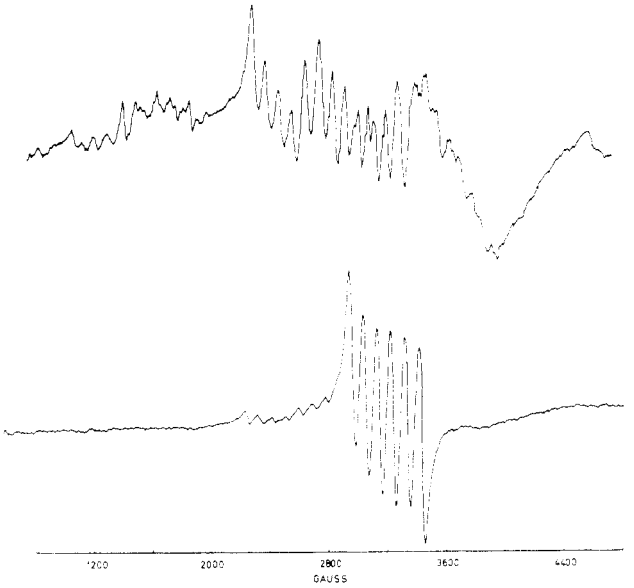

FIGURE 2: 9-GHz ESR spectra of Mn^{2+} bound to RuBP carboxylase. A solution of E–CO_2–Mn^{2+}–CPBP was prepared as described in Table II and isolated free of unbound components by Sephadex G-75 chromatography. The sample was concentrated to 1 mM prior to spectral measurements. In observing the undenatured complex (top spectrum), ESR measurements were performed at 0.5 °C, using a 10-G modulation amplitude. The bottom spectrum, recorded at a threefold lower gain setting, was observed after the initial sample was brought to 1% $NaDodSO_4$ and incubated at 23 °C for 15 h.

spectrum (vide infra) and is distinguishable from the much simpler and more intense spectrum of free hexaaquo Mn^{2+} (Knowles et al., 1976). Mn^{2+} in the enzyme complex was shown to be virtually nonexchangeable, regardless of whether exchange was measured as a decrease in the ESR signal of bound Mn^{2+} or as an increase in the ESR signal of free Mn^{2+}. A similar experiment was performed with $^{57}Co^{2+}$, which was incorporated stoichiometrically into E–CO_2–$^{57}Co^{2+}$–CPBP. The complex was isolated by gel filtration prior to mixing with a 100-fold excess of unlabeled $^{59}Co^{2+}$. After a 2-h incubation at 30 °C, the mixture was again subjected to gel filtration, and the stoichiometry of $^{57}Co^{2+}$ bound per active site was determined. As indicated in Table III, there was virtually no cation exchange, suggesting a remarkably stable interaction of cation with the other components in the complex.

The nonexchangeability of bound cation could be explained if a conformational change upon complex formation resulted in occlusion of the metal ion, so that rapid exchange with the components in the aqueous medium was prevented. Alternately, it is possible that cation is coordinated to other components of the complex in such a way that there is a negligible rate constant for dissociation of E–CO_2–M^{2+}–CPBP. This possibility suggested that direct observation of the bound cation would be of interest. E–CO_2–Mn^{2+}–CPBP was prepared, isolated free of unbound components, and concentrated to 1 mM enzyme sites. The ESR spectrum was recorded at 9 GHz by using conditions of high microwave power and modulation amplitude (cf. Experimental Section) and maintaining the sample at low temperature but in a liquid phase, as suggested by Reed & Ray (1971). As indicated in Figure 2, a complex spectrum is observed, not unlike those previously reported for Mn^{2+} bound to phosphoglucomutase (Reed & Ray, 1971) or to glutamine synthetase (Villafranca et al., 1976) but showing more structure downfield from the major peaks. The spectrum can be qualitatively explained by postulating that there is a large zero-field splitting for Mn^{2+} in the complex. This results in a shift of energy levels that accounts for fine structure

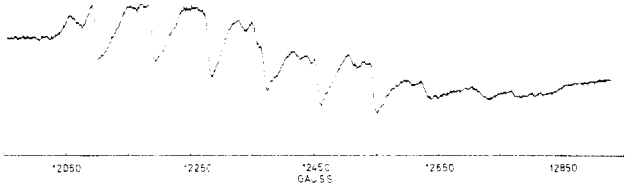

FIGURE 3: 35-GHz ESR spectrum of Mn^{2+} bound to RuBP carboxylase. A 1 mM solution of E–CO_2–Mn^{2+}–CPBP was prepared and isolated as described in Table II and loaded in a capillary for examination by ESR. Measurements were performed at 0.5 °C, using a 10-G modulation amplitude.

transitions which produce spectral lines displaced from the positions expected for the sextet that characterizes the Mn^{2+} hexaaquo ion. ion. The observed spectrum is compatible with a rhombic distortion of the coordination sphere of Mn^{2+}, which is evidently due to a change of Mn^{2+} ligands upon formation of the E–CO_2–Mn^{2+}–CPBP complex. An assignment of these ligands to the macromolecular or small molecule components of the complex cannot be made on the basis of these data. Additional experiments, in progress, may permit further characterization of the complex by an ESR approach.

The E–CO_2–M^{2+}–CPBP complex is labilized by $NaDodSO_4$ treatment (Miziorko, 1979). When a 1 mM sample of E–CO_2–Mn^{2+}–CPBP was brought to 1% in $NaDodSO_4$, the immobilized Mn^{2+} spectrum slowly collapsed, producing a signal (Figure 2, bottom) in which most of the sites show some increase in symmetry of the Mn^{2+} coordination sphere. The stability of the complex is considerable, however, since even after prolonged incubation of sample under these drastic conditions, elements of the immobilized spectrum are observable downfield from the major peaks. Also, the line widths of the major peaks are greater than those that characterize hexaaquo Mn^{2+}. These findings are, therefore, compatible with the observation of small but measurable levels of bound CO_2 isolated with enzyme after $NaDodSO_4$ denaturation of the complex (Miziorko, 1979).

A 1 mM sample of E–CO_2–Mn^{2+}–CPBP was also investigated at 35 GHz (Figure 3). At higher fields, a simpler spectrum might be expected since (a) the $-^1/_2$ to $+^1/_2$ spin transition should dominate, (b) the forbidden transitions, which could account for broadening at 9 GHz, should be less intense, and (c) spectral lines should be narrower if the electron spin relaxation time is field dependent. A simpler spectrum with narrower spectral lines in indeed observed, but the spectrum remains sufficiently complex to preclude detailed analysis at this time. The marked distortion of the symmetry of bound Mn^{2+} is likely to be associated with an unusual inner sphere liganding of the cation. If, in order to account for the nonexchangeability of cation demonstrated earlier (Table III), a choice had to be made between occlusion of bound cation from the bulk of the aqueous medium and complex inner sphere liganding of bound cation, the latter possibility seems to provide the simplest explanation compatible with all of the experimental data. However, a contribution from both of these two mechanisms cannot be ruled out.

Discussion

The ability of carboxypentitol bisphophate to inactivate RuBP carboxylase by forming an exceptionally stable complex permitted Lane and his colleagues to partially characterize the model complex and to predict details of the mechanism whereby a six carbon carboxylated intermediate, 2-carboxy-3-ketopentitol bisphosphate, could be produced and cleaved to form the products of the overall reaction (Wishnick & Lane, 1969; Wishnick et al., 1970; Siegel & Lane, 1972, 1973).

However, the claim that CO_2 did not affect CPBP inhibition (Siegel & Lane, 1972) was difficult to reconcile with subsequent observations. There was evidence that irreversible enzyme inhibition by CPBP required participation of a cation, and Miziorko & Mildvan (1974) established that no specific binding of cation by RuBP carboxylase would occur in the absence of CO_2. The detection of an activated enzyme–CO_2–M^{2+} complex prompted the idea that CPBP binds to this species, and, thus, a reexamination of the composition of the stable inhibited complex seemed warranted.

Recently, it was demonstrated that both CO_2 and CPBP are bound in the same enzyme complex (Miziorko, 1979). Such an observation is in accord with the requirement for CO_2 to promote cation binding which, in turn, supports CPBP binding and a stabilization of the complex which is described in this report. Since elements of CO_2 activator and CO_2 substrate are both present in the E–CO_2–M^{2+}–CPBP complex, a question arises concerning cation participation in activation and/or catalysis. Laing & Christeller (1976) observed that, upon dilution of active E–CO_2–Mg^{2+} into assay mixtures containing variable levels of Mg^{2+}, the measured reaction rate was independent of total Mg^{2+}. They concluded that catalysis is independent of cation concentration. This argument involves the assumption that bound cation in the ternary E–CO_2–M^{2+} complex dissociates rapidly upon dilution of activated enzyme into the assay mixture. If RuBP behaves analogously to CPBP, metal dissociation in the presence of substrates may not be very rapid. Thus, the possibility of a slowly exchanging cation complicates interpretation of those data. If the cation is, in fact, involved only in activation of enzyme by stabilizing carbamate formation, the simple model shown in Figure 1 may be inadequate to account for what is observed when substrate sites are occupied (e.g., by the transition-state analogue, CPBP). The nonexchangeability of cation observed under those conditions suggests that there are additional ligands which contribute extra stability to the E–CO_2–M^{2+} adduct.

The reports of varying rates of carboxylation in the presence of different cations (Wildner & Henkel, 1978, 1979) may offer an indirect argument supporting a role for cation in catalysis. Since it is possible to lock in a variety of cations on RuBP carboxylase (Table II; Wishnick et al., 1970) while stoichiometrically trapping the CO_2 activator, a reasonable explanation for variable catalytic efficiency of enzyme that is saturated with activators is that different cations have different efficacy in supporting carboxylation. Cation participation in catalysis is also suggested by the observation that bound Mn^{2+} is in close proximity to a rapidly exchanging CO_2 (Miziorko & Mildvan, 1974). If CO_2 activator actually forms, upon binding to enzyme, a carbamate which is stable enough to be detected by NMR (O'Leary et al., 1979), this suggests that the rapidly exchanging CO_2 monitored in the earlier NMR relaxation experiments was the substrate and that cation is, therefore, close to the catalytic site. Any assignment of a catalytic role to cation must be based, at this point, on indirect evidence. However, since the stoichiometry of metal binding in E–CO_2–M^{2+}–CPBP approaches 1, it would seem that, if there is, in fact, involvement of cation in catalysis, the cation involved in forming the active ternary complex would have to be functioning in both capacities. Experiments aimed at a more direct observation of the activators and substrates are needed to provide a test of the schemes for activation and catalysis and to help in explaining the distortion in environment of the bound cation activator. Physical and chemical studies employing the E–CO_2–Mn^{2+}–CPBP model complex are currently in progress and are aimed at improving our understanding of the detailed mechanism of RuBP-dependent CO_2 fixation.

Acknowledgments

We are grateful to the National Biomedical ESR Center (Milwaukee, WI) for making available the ESR spectrometers used in these studies and to Dr. Neil Maher (West Allis Memorial Hospital Laboratory) for performing the atomic absorption analyses.

References

Andrews, T. J., Lorimer, G. H., & Tolbert, N. E. (1973) *Biochemistry 12*, 11–18.

Copper, T. G., Filmer, D. L., Wishnick, M., & Lane, M. D. (1969) *J. Biol. Chem. 244*, 1081–1083.

Knowles, P. F., Marsh, D., & Rattle, H. W. E. (1976) *Magnetic Resonance of Biomolecules*, p 200, Wiley, New York.

Laing, W. A., & Christeller, J. T. (1976) *Biochem. J. 159*, 563–570.

Lorimer, G. H. (1979) *J. Biol. Chem. 254*, 5599–5601.

Lorimer, G. H., Badger, R. R., & Andrews, T. J. (1976) *Biochemistry 15*, 529–536.

Lorimer, G. H., Badger, M. R., & Andrews, T. J. (1977) *Anal. Biochem. 78*, 66–75.

Miziorko, H. M. (1978) *Life Sci. 11*, 41–42.

Miziorko, H. M. (1979) *J. Biol. Chem. 254*, 270–272.

Miziorko, H. M., & Mildvan, A. S. (1974) *J. Biol. Chem. 249*, 2743–2750.

O'Leary, M. H., Jaworski, R. J., & Hartman, F. C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 673–675.

Paulsen, J. M., & Lane, M. D. (1966) *Biochemistry 5*, 2350–2357.

Pierce, J., Tolbert, N. E., & Barker, R. (1979) *Abstracts of Papers*, National Meeting of the American Chemical Society, Washington, D.C., Sept 1979, American Chemical Society, Washington, D.C., BIOL 2.

Reed, G. H., & Ray, W. J. (1971) *Biochemistry 10*, 3190–3197.

Robison, P. D., Martin, M. N., & Tabita, F. R. (1979) *Biochemistry 18*, 4453–4458.

Siegel, M. I., & Lane, M. D. (1972) *Biochem. Biophys. Res. Commun. 48*, 508–516.

Siegel, M. I., & Lane, M. D. (1973) *J. Biol. Chem. 248*, 5486–5498.

Siegel, M. I., Wishnick, M., & Lane, M. D. (1972) *Enzymes, 3rd Ed. 6*, 169–192.

Villafranca, J. J., Ash, D. E., & Wedler, F. C. (1976) *Biochemistry 15*, 544–553.

Weissbach, A., Horecker, B. L., & Hurwitz, J. (1956) *J. Biol. Chem. 218*, 795–810.

Wildner, G. F., & Henkel, J. (1978) *FEBS Lett. 91*, 99–103.

Wildner, G. F., & Henkel, J. (1979) *Planta 146*, 223–228.

Wishnick, M., & Lane, M. D. (1969) *J. Biol. Chem. 244*, 55–59.

Wishnick, M., Lane, M. D., & Scrutton, M. C. (1970) *J. Biol. Chem. 245*, 4939–4947.