The Orientation of Substrate and Reaction Intermediates in the Active Site of Ribulose-1,5-bisphosphate Carboxylase*

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There are four possible orientations of the substrate ribulose 1,5-bisphosphate in the active site of ribulose 1,5-bisphosphate carboxylase. Distinction between these four possible orientations has been made on the basis of $^{31}$P NMR and borohydride-trapping experiments.

The orientation of the reaction-intermediate analog, 2'-carboxy-D-arabinitol 1,5-bisphosphate with respect to the divalent metal ion was determined by $^{31}$P NMR studies of the quaternary complex, enzyme-CO$_2$Ni$^{2+}$-2'-carboxyarabinitol 1,5-bisphosphate. Assignment of the phosphorus resonances of this complex was made by labeling the phosphoryl group at either C-1 or C-5 with $^3$O. The phosphorus atom closer to the divalent metal ion has been annotated as that attached to C-1.

When bound to the active site of carboxamidated enzyme, D-ribulose 1,5-bisphosphate was reduced by sodium borohydride with absolute stereospecificity to D-ribitol 1,5-bisphosphate. The reduction of the enzyme-bound substrate thus occurred on the Si face of the C-2 carbonyl group.

These two results together establish that ribulose 1,5-bisphosphate is oriented within the active site so that 1) the phosphoryl group at C-1 is closer to the divalent metal ion than that at C-5 and 2) the Si face of the carbonyl group points to the "outside world."

Recently, the three-dimensional structures of three forms of ribulose-P$_2$ carboxylase$^1$ have been solved to 2.8 Å resolution; the decarboxamidated dimer from Rhodospirillum rubrum (Schenider et al., 1986), the decarbaminated hexadecamer from tobacco (Chapman et al., 1988), and the quaternary complex, E.4CO$_2$Mg-CABP from spinach (Andersson et al., 1989). The same structural motif is found in both dimeric and hexadecameric ribulose-P$_2$ carboxylases, namely, an eight-stranded α/β barrel at the carbonyl end of which the active site is located. The current stage of refinement of the spinach quaternary complex, three "landmarks" within the active site may be defined with certainty. The position of the divalent metal ion has been determined by Fourier difference techniques using the cobalt-substituted enzyme. It is coordinated to the carbamate group on the ε-amino group of Lys-201. Additionally, two regions of well defined electron density are apparent. These correspond to the two phosphoryl groups of CABP and are 9.6 Å apart (Andersson et al., 1989). We define the binding sites for these phosphoryl groups as A and B (Fig. 1), without specifying to which site the two phosphoryl groups, the one at C-1 and the other at C-5, bind.

The remainder of the CABP molecule is poorly defined, leaving its stereochemical orientation in question. Site A, centered upon Gly-394 of loop 8, is located 6.6 Å from divalent metal ion, while site B, centered upon Arg-288 of loop 5, is 7.8 Å removed from the divalent metal ion. These distances are large enough to preclude the direct coordination of the divalent metal ion with either of the two phosphoryl groups. The ratio of these crystallographically determined distances, 1.19, agrees rather well with the value of 1.2, determined previously by $^{31}$P NMR methods (Pierce and Reddy, 1986).

The Substrate Docking Problem—Before consideration can be given to the mechanistic involvement of the various functional groups within the active site, it is imperative that the orientation of the substrate and reaction intermediates within the active site be determined with certainty. With the substrate ribulose-P$_2$, there are two independent problems of orientation to be solved.

The first problem arises from the presence of the two phosphoryl groups at opposite ends of the molecule. Given the presence of two phosphoryl binding sites A and B in the active site, there are then two orientations of the substrate to be considered. Either binding site A accommodates the C-1 phosphoryl group and binding site B accommodates the C-5 phosphoryl group (Fig. 1, II and IV) or vice versa (Fig. 1, I and III).

The second problem of orientation arises from the planar nature of the C-2 carbonyl group. Here again, two orientations need to be considered. Either the Si face of the carbonyl group (Fig. 1, III and IV) or the Re face (Fig. 1, I and II) points to the outside world. In this report we describe the results of experiments specifically designed to address the substrate docking problem.

Orientation of the Phosphoryl Groups at C-1 and C-5 of CABP. Which Is Closer to the Divalent Metal Ion?—In an earlier study Pierce and Reddy (1986) reported the $^{31}$P NMR spectra of the quaternary complexes E.4CO$_2$.Me-CABP prepared with Mg$^{2+}$ and a variety of paramagnetic metal ions (Mn$^{2+}$, Co$^{2+}$, and Ni$^{2+}$). In free solution at pH 8.1, CABP displayed two sharp resonances at 1.6 and 2.0 ppm, relative to orthophosphate at 0 ppm (see Fig. 3). When CABP was incorporated into the Mg$^{2+}$-quaternary complex, these resonances were so broadened that a single, unresolved resonance

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1 The abbreviations used are: ribulose-P$_2$, carboxylase, ribulose-1,5-bisphosphate carboxylase/oxygenase; pen-titol-P$_2$, the unresolved mixture of ribitol 1,5-bisphosphate and arabinitol 1,5-bisphosphate; arabinitol-P$_2$, arabinitol 1,5-bisphosphate; ribitol-P$_2$, ribitol 1,5-bisphosphate; CO$_2$, the molecule of CO$_2$ which becomes a carbamate upon activation of ribulose-P$_2$ carboxylase; ribulose-P$_2$, ribulose 1,5-bisphosphate; CABP, 2'-carboxy-D-arabinitol 1,5-bisphosphate; CRBP, 2'-carboxy-D-ribitol 1,5-bisphosphate; HPLC, high performance liquid chromatography; Bicine, N,N-bis(2-hydroxyethyl)glycine.
Ribulose-P₂ Carboxylase: Orientation of the Substrate

**I**

1B, 5A : Re

1, 5B : Re

**II**

1A, 5B : Re

**III**

1B, 5A : Si

1, 5B : Si

**IV**

1A, 5B : Si

**FIG. 1.** The four possible orientations of the substrate within the active site of ribulose-P₂ carboxylase. The active site is located at the carboxyl end of an eight-standard α/β barrel (Andersson et al., 1988). The active site residues are found on unstructured “loops” which connect the carboxyl ends of the β-strands with the amino ends of the α-helices. These loops are represented here as the sides of an octagon and are so numbered. Thus, for example, residues contributing to the phosphoryl binding sites A and B are found on loops 8 and 5, respectively, while the activator Lys-201 (spinach numbers) is found on loop 2. The active site is seen from the perspective of an incoming substrate molecule as it attempts to dock. In the current electron density map three landmarks are evident: the phosphoryl binding site A, the phosphoryl binding site B, and the divalent metal ion Me. Note that site A is closer to Me than site B. Two independent problems of orientation arise for which there are two solutions each. 1) Which phosphoryl binding site, A or B, binds which phosphoryl group? Site A binds either the C-1 phosphoryl group (orientations ZZ and ZZ') or the C-5 phosphoryl group (orientations Z and ZZZ). 2) Which face of the C-2 carbonyl group points to the “outside world”; the Re face (as in orientations Z and ZZ) or the Si face (as in orientations III and IV)? The stereochemical orientation of the hydroxyl groups at C-3 and C-4 are arbitrarily chosen.

was observed (Pierce and Reddy, 1986). But when the quaternary complex was prepared with paramagnetic Ni²⁺, these unresolved resonance signals were shifted and differentially broadened because of the relaxation effects of the paramagnetic Ni²⁺. This differential broadening of the phosphorus resonances indicated that one of the phosphorus atoms is slightly closer to the metal ion than the other and permitted the distance ratio, phosphorus to metal, of 1.2 to be computed. In this study the differentially broadened resonances of the Ni²⁺-quaternary complex could not be assigned to a particular phosphorus atom. Thus the question as to which of the two phosphorus atoms is closer to the metal ion, which would define the orientation of the substrate within the active site, was left unanswered.

When ¹⁷O is directly bonded to ³¹P; a rapid relaxation mechanism is provided which leads to the extreme broadening of the ³¹P resonance. Ryu et al. (1984) have exploited this phenomenon to assign the ³¹P resonances of phosphoglucomutase to which ¹⁷O-labeled ligands (P₃ or glucose phosphate) were bound. This suggested to us that ¹⁷O labeling of each of the phosphoryl groups of CABP could be used to assign the ³¹P NMR peaks of both free CABP and of CABP bound to ribulose-P₂ carboxylase as part of a E·AC02.Ni.CABP quaternary complex. From such an assignment we could then specify which of the two phosphoryl groups, that at C-1 or that at C-5, is closer to the divalent metal ion, which in turn would define the orientation of the substrate within the active site.

Orientation of the Carbonyl Groups at C-2 of Ribulose-P₂: Which Face (Re or Si) Points to the Outside World?—The orientation of the substrate carbonyl group within the active site of several enzymes has been successfully determined by borohydride reduction. The attack of borohydride on the enzyme-bound substrate has been shown to be stereospecific for pyruvate kinase (Kosicki and Westheimer, 1968; Phillips et al., 1973; Creighton and Rose, 1976), triose phosphate...
isomerase (Webb and Knowles, 1974, 1975) and fructose-1,6-
bisphosphate aldolase (Di Tasio et al., 1977). These enzymes
share a common structural motif, an eight-stranded α/β barrel
at the carboxyl end of which the active site is located (Schnei-
der et al., 1986; Banner et al., 1975; Stuart et al., 1979; Sygusch
et al., 1987). These facts suggested that the orientation of the
C-2 carbonyl group of ribulose-Pz within the active site might
determine by the borohydride reduction method.

In Fig. 2 the stereochemical alternatives with the reduction of ribulose-Pz are shown. Attack of borohydride
on the Si face leads to the formation of D-arabinitol 1,5-
bisphosphate, while attack on the Re face results in R-arabitol
1,5-bisphosphate. Here we report that when ribulose-Pz is
reduced by borohydride while bound to the active site of
carboxaminated ribulose-Pz carboxylase, the product is exclu-
sively D-arabinitol 1,5-bisphosphate. Consequently, ribulose-
Pz is oriented in the active site in such a manner that the Si
face of the carbonyl group points to the outside world.

A preliminary account of parts of this work was presented
at the Sixth International Congress of Photosynthesis, 1983
(Lorimer et al., 1984).

**EXPERIMENTAL PROCEDURES**

**Materials**

Ribulose-Pz carboxylases were purified from the following sources as previously described: spinach (Lorimer et al., 1976) and Escherichia coli
expressing a plasmid-encoded gene from *Rhodospirillum rubrum*
(Pierce and Gutteridge, 1985; Pierce and Reddy, 1986). Ribulose-Pz
and CABP were synthesized and purified by published methods
(Horecker et al., 1958; Pierce et al., 1980). [U-14C]Ribulose-Pz was
prepared by coupled enzymatic synthesis from [U-14C]glucose.
The concentrations of spinach and *R. rubrum* enzymes were measured
using published extinction coefficients (Paulsen and Lane, 1966;
Schloss et al., 1982).

**Methods**

Preparation of 170-Labeled Orthophosphate—170-Labeled ortho-
phosphate was prepared by a modification of the method of Risley
and Van Etten (1978). Following the reaction of 170-labeled water
with PCl3, HCl was removed by evaporation to dryness under vacuum.
NaOH was added to the residual [170]PO4 at a final pH of 8.1 and the
solution passed through a 6 × 1 cm column of Chelex 100 resin (Bio-
Rad). 31P NMR analysis showed that [170]PO4 amounted to 11.7%
of the total P.

Preparation of [1-160,5-170]Carboxyarabinitol 1,5-Bisphosphate—
6-[1-170,5-160]Phosphoglucoconate was first prepared by reacting,
in a total volume of 25 ml, 44 µmol of [1-170]P, 1 mmol of sucrose, 0.5
mmol of MgCl2, 0.5 µmol of glucose 1,6-bisphosphate, 373 µmol of
NADP+, and 1.36 mmol of triethanolamine·Cl, pH 7.0, with 7.2 units
of glucose-6-phosphate dehydrogenase. The reaction was monitored
at 340 nm. After 2 h at room temperature 2.5 g of washed, active charcoal were added to remove nucleotides.
The charcoal was removed by centrifugation. To the supernatant
(plus washings) was added 320 µmol of NADP+, 200 µmol of MgCl2,
and sufficient NaOH to bring the pH to 7.5. To this was added 19
units of 6-phosphogluconate dehydrogenase. The mixture was
incubated at 340 nm, when the conversion of 6-
phosphogluconate to ribulose 5-phosphate was complete, 1 mmol of
dithiothreitol and 313 µmol of ATP were added, and the pH was
adjusted to 7.7 before the addition of 33 units of phosphoribulokinase
kinase. Formation of [1-160,5-170]ribulose-Pz was monitored by ana-
lyzing aliquots by the method of Andrews and Lorimer (1986). When
the kinase reaction was complete, nucleotides were removed by the
addition of 7.5 g of washed, active charcoal and centrifugation.
[1-160,5-170]Ribulose-Pz was recovered from the supernatant plus
washings by precipitation as the barium salt from 50% ethanol.
The insoluble barium salt was washed once with 70% ethanol and the
precipitate recovered by centrifugation. The precipitate was dissolved
by the addition of a suspension of Dowex 50 (H+ form) resin. The
filtrate plus washings were adjusted to pH 8.4 by the addition of 1 M
Tris base. To this was added a 2-fold molar excess KCN to a final
pH of 8.7. After 30 h room temperature, a suspension of Dowex 50
(50% ethanol) was added, and the filtrate plus washings were
allowed to stand overnight at room temperature to affect saponifica-
tion.

31P NMR analysis (Fig. 3B) of the resultant [1-160,5-170]CABP
revealed the presence of non-170 in the 5-position. Since the bridge
oxygen is unlabeled, the expected fraction of non-170 in the [5-170]
CABP synthesized from [1-160,5-170]Pi was calculated to be 0.117
(0.585)3 = 0.117.

Preparation of [1-160,5-170]Ribulose-Pz—170-Labeled ribulose-Pz was first prepared by reacting, in a total
volume of 45 ml, 56 µmol of NADP+, 73 µmol of ADP, 285 µmol
of fructose 1,6-bisphosphate, 1 mmol of sodium pyruvate, 1 mmol
of MgCl2, 526 µmol of ribose 5-phosphate, 400 µmol of dithiothreitol,
750 units of ribose 5-phosphate isomerase, 320 units of phosphoribulokinase, 780 µmol of [1-160]P, 90 units of fructose 1,6-
bisphosphate aldolase, 680 units of triose phosphate isomerase, 200
units of glyceraldehyde 3-phosphate dehydrogenase, 530 units of 3-
phosphoglycerate kinase, and 450 units of lactate dehydrogenase. The
pH of the reaction mixture was maintained at 7.8 by the addition of
NaOH. This permitted the progress of the reaction to be monitored.
{(One equivalent of NaOH is consumed per mol of ribulose-Pz formed.)
After the consumption of 500 meq of NaOH (95% reaction based on
ribose 5-phosphate), nucleotides were removed by the addition of 2.5
mmol of washed, active charcoal. The [1-160,5-170]ribulose-Pz was
recovered as the barium salt from 50% ethanol and further purified by
chromatography on a 1 × 9 cm column of Dowex 1-X8 (Cl form)
equilibrated with 50 mM sodium acetate, 10 mM NaCl, pH 4.5. The
column was developed with a 350 ml of linear gradient to 1 M NaCl
in 50 mM sodium acetate, pH 4.5. The eluate was monitored at 280
nm and fractions containing ribulose-P2 combined and recovered as
the barium salt from 50% ethanol. The barium salt was washed once
with 70% ethanol. The procedure for preparing [1-160,5-170]CABP
from this point onward was identical to that described above for
[1-
160,5-170]CABP.

31P NMR analysis (Fig. 3C) of the resultant [1-160,5-170]CABP
reveal the presence of non-170 in the 1-position. Although sufficiently
low for our purpose, the fraction of non-170 was larger than the 0.20
expected from [1-160,5-170]Pi.

Preparation of E-Co2+-Ni-CABP Quaternary Complex—A modifi-
cation of the procedure described by Pierce and Reddy (1986) was
used. To about 6 mmol of *R. rubrum* carboxylase protein in about
5 ml of 50 mM Tris-Cl, pH 8.1, was added 240 µl of 1 M NaHCO3 and

5 There is reason to believe that at least one of the enzymes used
to convert [1-170]P to [1-170]ATP was contaminated with carboxylic
kinase. This would decrease the isotopic enrichment of the terminal
phosphoryl group which was subsequently transferred to the 1-posi-
tion of ribulose-Pz and hence to CABP.
250 µl of 71.5 mM NaCl (a 2.7-fold molar excess over protomer). After a further 45 min. The sample was then applied to a Sephadex G-50, equilibrated at 4 °C with 50 mM Tris-Cl, pH 8.1. Fractions containing the quaternary complex were combined and HzO to 15% was added. The quaternary complex was then concentrated to about 1 mM in protomer on an Amicon PM-30 membrane. A slight turbidity was removed by centrifugation prior to 31P NMR analysis.

**NMR Spectroscopy—**31P NMR spectra of “free” CABP were obtained at 146 MHz with broadband proton decoupling in spinning 12-mm tubes at 25 °C. The recycling time was 3.6 s. Samples contained 15% HzO for the H field-frequency lock. A small amount of orthophosphate (pH 8.1) was added to serve as an internal chemical shift reference at 0 ppm. Transients were accumulated.

31P NMR spectra of the quaternary complexes were obtained at 146 MHz without broadband proton decoupling in nonspinning 12-mm tubes at 5 °C. The recycling time was at least 200 ms. Samples contained 15% HzO. The spectra were referenced to a spectrum of orthophosphate, pH 8.1. recorded immediately after the test spectrum without changing the field or lock parameters. At least 10,000 transients were recorded. A line-broadening factor of 20 Hz was applied to the experimental spectra.

**Borohydride Reduction of Ribulose-1,5-Bisphosphate: Rapid Mixing and Quenching—**This was performed using a chemical quench apparatus (System 1000 equipped with a Model 705A controller from Update Instruments Inc., Madison, WI). Details of reaction solutions, reaction times, and the composition of the quenching solutions are provided in the figure legends. The general protocol for mixing and quenching was as follows. Enzyme and radioactive ribulose-Ps, delivered from separate syringes, were combined in the mixing chamber and allowed to react for a predetermined time. The mixture was then delivered to a solution of 0.125 M triethanolamine-HCl, pH 8.5, containing NaBH4 at the indicated concentration. The resultant solution was allowed to stand for about 30 min at 24 °C before analysis of the pentitol bisphosphates.

**Analysis of the Pentitol 1,5-Bisphosphates—**The solutions containing the pentitol bisphosphates were first adjusted to pH 5.3 with 10% (v/v) formic acid. The precipitated protein was removed by centrifugation. The supernatant solution was treated with 5-10 g of Dowex 50 (H+ form) and the resin removed by filtration. The filtrate plus washings were evaporated to dryness repeated form acidic methanol to remove borate. The residue was dissolved with 10 ml of a solution containing 1 M Tris-Cl, pH 9.5, 1 mM MgCl2, 0.1 mM ZnSO4, 0.05 mM EDTA. The pH was adjusted to 9.5 (if necessary) with NH4OH before adding about 10 units of alkaline phosphatase. Hydrolysis of the phosphate esters was allowed to proceed overnight at 24 °C. The solution was then passed successively through 0.7 and 5-cm columns of Dowex 50 (H+ form) and Dowex 50 (H+ form). The filtrate plus washings were combined and reduced to dryness in vacuo. The residue was dissolved with 0.1 ml of a solution containing 10 mg/ml each of authentic D-ribitol and D-arabinitol. Aliquots of this solution were injected on to a 500 x 7.8-mm Bio-Rad Aminex Carbohydrate HPZ-57C column equilibrated at 85 °C with water. The peaks of radioactive pentitols were collected using the refraction of the carrier pentitols as a guide. More than 80% of the radioactivity injected on to the column was recovered in the two peaks corresponding to ribitol and arabinitol (Fig. 5). Radioactivity was measured by scintillation counting using the cross-channel ratio method to correct for quenching.

**RESULTS AND DISCUSSION**

**Orientation of the Phosphoryl Groups—**The 31P NMR spectra of free CABP revealed two sharp peaks at 1.6 and 2.0 ppm (Fig. 3A). These peaks were of equal intensity and their linewidths (1.3 Hz) were identical. In order to assign the two 31P NMR peaks, [1-'70,5-'70]CABP and [1-'60,5-'60]CABP were prepared. The spectra are shown in Fig. 3, B and C. Labeling of either phosphorus atom with one 17O nucleus was sufficient to broaden the 31P NMR peak beyond detection. On this basis, the peaks at 1.6 and 2.0 ppm were assigned to the phosphoryl groups at positions 1 and 5, respectively. The residual intensities observed at 1.6 and 2.0 ppm in the spectra of [1-'70,5-'70]CABP and [1-'60,5-'60]CABP, respectively, can be attributed to phosphorus atoms to which no 17O is bonded. Based upon the fraction of 17O present in the orthophosphate used to prepare the enriched CABPs (0.42), 20% of the phosphorus atoms at position 5 of [1-'70,5-'70]CABP and at position 1 of [1-'70,5-'70]CABP were predicted to be devoid of 17O. Experimentally, we observed that 16% of the phosphoryl groups at position 5 of [1-'70,5-'70]CABP lacked 17O. The theoretical and experimental intensities are probably
within experimental error. In the case of $[^1-17O,5-16O]\text{CABP}$, some 36% of the phosphoryl groups at position 1 were devoid of $^{17}O$. Although the $^{17}O$ enrichment at position 1 of $[^1-13O,5-18O]\text{CABP}$ was much less than predicted, it was nevertheless sufficient for the purpose of assignment.

The $^{31}P$ NMR spectrum of the Ni$^{2+}$-quaternary complex prepared with unenriched $[^1-16O,5-16O]\text{CABP}$ (Fig. 4B) was identical to that previously reported (Pierce and Reddy, 1986). As before, the two phosphorus resonances of the enzyme-bound CABP were both shifted and differentially broadened in the presence of the paramagnetic Ni$^{2+}$ ion. The higher field resonance at $-4 \text{ ppm}$ had a linewidth of about 120 Hz, while the lower field resonance at about 1 ppm had a linewidth of about 60 Hz. However, one cannot assign these resonances to a specific phosphorus atom because of uncertainty in the directionality of the induced paramagnetic shifts. In order to assign these resonances, the quaternary Ni$^{2+}$ complexes, prepared with $[^1-17O,5-16O]\text{CABP}$ or with $[^1-16O,5-17O]\text{CABP}$, were analyzed (Fig. 4, A and C). Note that in both cases, the unenriched phosphorus atom of the pair served as an internal reference. Thus, a comparison could be made between the spectrum of a $^{16}O$-enriched quaternary complex (spectra A or C of Fig. 4) and the spectrum of the control, unenriched 1-18O,5-16O-labeled quaternary complex (spectrum B of Fig. 4).

Broadening of one or the other phosphorus resonance, attributable to $^{17}O$-labeling, was evident in the spectra of both enriched complexes. In the case of the complex prepared with $[^1-17O,5-17O]\text{CABP}$, broadening of the higher field resonance was evident (Fig. 4A). However, the linewidth of the lower field resonance (about 60 Hz) was similar to that observed in the complex prepared with $[^1-16O,5-16O]\text{CABP}$. Analysis of the reciprocal complex prepared with $[^1-16O,5-17O]\text{CABP}$ (Fig. 4C) yielded a result consistent with that above. In this instance, the lower field resonance was even more strikingly broadened by $^{17}O$-labeling. The linewidth of the higher field resonance (about 120 Hz) was similar to that observed in the complex prepared with $[^1-16O,5-16O]\text{CABP}$, however. On the basis of these results, the higher field resonance was assigned to the phosphoryl group at C-1, while the lower field resonance was assigned to the phosphoryl group at C-5. In the spectrum of the unenriched complex (Fig. 4B) the higher field resonance due to the C-1 phosphoryl is broader than the lower field resonance due the C-5 phosphoryl. This differential broadening can be attributed to the closer proximity of the C-1 phosphorus nucleus to the paramagnetic Ni$^{2+}$ ion; i.e. the phosphoryl group at C-1 is closer to the metal ion than the phosphoryl group at C-5. In terms of the four possible orientations of the substrate shown in Fig. 1, the orientations I and III may therefore be discounted. Distinguishing between the remaining orientations II and IV requires that the orientation of the C-2 carbonyl group with respect to the "outside world" be determined.

The Orientation of the C-2 Carbonyl Group—Reduction of ribulose-P$_2$ in free solution yielded the expected mixture of ribitol-P$_2$ and arabitol-P$_2$. Following treatment with alkaline phosphatase, the resultant pentitols were fully resolved from one another and from xylitol by high pressure liquid chromatography (Fig. 5B). The reduction of ribulose-P$_2$ in free solution occurred with a slight stereocchemical preference for attack from the Re face of the carbonyl group, producing a ribitol:arabitol ratio of 1.3:1. When the reduction of ribulose-P$_2$ by borohydride was conducted in the presence of an approximately 3-fold molar excess (based on protomer) of activated carboxylase, containing the Lys-201-carbamate-Mg$^{2+}$ complex, the results shown in Fig. 5C were obtained. The predominance of arabinitol over ribitol (in this instance by a ratio of 7:1) established that there was a distinct stereochemical preference for reduction to occur from the Si face of the substrate shown in Fig. 1, the orientations I and III may therefore be discounted. Distinguishing between the remaining orientations II and IV requires that the orientation of the C-2 carbonyl group with respect to the "outside world" be determined.
Clearly the stereospecific reduction of ribulose-P₂ by borohydride in the presence of the activated enzyme occurred within the active site.

The degree of stereospecificity of reduction of enzyme-bound substrate was next addressed by performing the reduction with varying substrate:enzyme ratios. The results are shown in Fig. 6. If the stereospecificity of reduction was absolute, it was to be expected that, when all of the substrate was bound, only arabinitol would be produced; i.e. a plot of \( \text{reduced arabinitol} \) versus \( \text{substrate:enzyme ratio} \) should pass through the origin. Within the limits of experimental error, the regression line passes through the origin. The degree of stereospecificity was examined as before. The result (Fig. 6) demonstrated that, in contrast to the reduction of ribulose-P₂ at the catalytic site of activated enzyme, that occurring at the catalytic site of deactivated enzyme was not absolutely stereospecific. The lack of complete stereospecificity of borohydride reduction evident when the substrate is bound to deactivated enzyme can be explained by the absence of the orienting effect of the divalent metal ion. For example, if the orientation of the rest of the substrate (e.g. the phosphoryl groups at C-1 and C-5) with respect to the enzyme remains the same in the absence of the carbonyl divalent metal ion interaction, the carbonyl group may be able to dock with either the \( \text{Re} \) or the \( \text{Si} \) face exposed to attack by borohydride.

Mechanistic Implications—When considered in conjunction with the crystallographic evidence (Andersson et al., 1989), the results reported here indicate that ribulose-P₂ is oriented in the active site in the manner represented by IV of Fig. 1. In this orientation the carbonyl oxygen is pointed more or less directly at the divalent metal ion. This interpretation is consistent with spectroscopic evidence (Mizioroko and Sealy, 1984; Styring and Branden, 1985) which indicates that the hydroxyl oxygen at C-2 of C2BP is directly coordinated to the divalent metal ion. Interaction of the divalent metal ion with the carbonyl group of the substrate would assist in the establishment of the stereochemically correct orientation of the substrate within the catalytic site. Additionally, polarisation of the carbonyl group by the divalent metal ion would facilitate abstraction of the proton at C-3 leading to formation of the enediol(ate). The subsequent reaction of the \( \text{Si} \) face of this enediol(ate) with the gaseous substrates, \( \text{CO}_2 \) or \( \text{O}_2 \), is internally consistent with the proposed ordered kinetic path-
way of catalysis (Pierce et al., 1986a, 1986b) and with the stereocchemical configuration about C-2 of the resulting 6-carbon reaction intermediate, 2-carboxy-3-ketoarabinitol-P$_2$ (Schloss and Lorimer, 1982).

Acknowledgments—The impetus for this study arose as a result of discussions with Prof. Carl Branden and his colleagues concerning uncertainties in the orientation of CABP within the active site of spinach ribulose-P$_2$ carboxylase. Their input enabled us to formulate the substrate docking problem in a more precise manner than would otherwise have been possible. We also thank Dr. John Pierce for his helpful comments regarding $^{31}$P NMR spectroscopy.

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