INTERACTION OF RIBULOSE DIPHOSPHATE CARBOXYLASE WITH 2-CARBOXY-RIBITOL DIPHOSPHATE, AN ANALOGUE OF THE PROPOSED CARBOXYLATED INTERMEDIATE IN THE CO_2 FIXATION REACTION.

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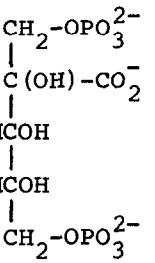
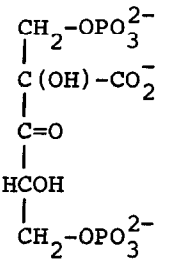
SUMMARY

Support for the participation of a six-carbon carboxylated intermediate (or transition state) in the D-ribulose-1,5 diphosphate carboxylase-catalyzed reaction was derived from kinetic and binding studies with 2-carboxy-D-ribitol-1,5-diphosphate (CRDP), an analog of the proposed intermediate and potent inhibitor of the reaction. Evidence is presented that following the formation of a carboxylase·Mg·CRDP complex, a slow temperature-dependent conformational change occurs locking the enzyme in an inhibited state from which release of the inhibitor is extremely slow.

Earlier investigations in this laboratory (1) have shown that 2-carboxy-D-ribitol-1,5-diphosphate (CRDP, Structure I), is a potent inhibitor of the ribulose diphosphate carboxylase-catalyzed reaction.



I.



II.

It was suggested (1) that CRDP acts as an inhibitor because of its structural similarity to the hypothetical carboxylated intermediate, 2-carboxy-3-keto-D-ribitol-1,5-diphosphate (Structure II) proposed by Calvin (2) or a closely-related transition state in the enzymatic reaction. Were this the case, it would be anticipated that the inhibitor would bind more tightly than either sub-

strate, i.e. ribulose diphosphate or CO_2, since it contains structural elements of both. The participation of either the proposed intermediate or transition state in the reaction is consistent with other findings on the enzymatic mechanism (3). The present communication provides evidence that CRDP acts at the active site and has the characteristics of an analogue of the hypothetical carboxylated six-carbon intermediate or transition state.

METHODS

Ribulose diphosphate carboxylase (specific activity, 1.2-1.5 units per mg) was prepared in homogeneous form from spinach leaves and assayed by the methods of Paulsen and Lane (4). Unlabeled and [carboxy-^{14}C] CRDP were chemically synthesized and chromatographically-purified as described earlier (1). Previous investigations showed that the carboxylase has a molecular weight of 5.6×10^5 (4), is composed of 8 pairs of nonidentical subunits (5,6) and binds 8 moles of D-ribulose-1,5-diphosphate or CRDP per molecule of enzyme (1).

RESULTS

Exposure of ribulose diphosphate carboxylase for 20-40 min to a 10-fold stoichiometric excess of CRDP over enzyme (a 1.25-fold excess over the 8 ribulose diphosphate substrate binding sites per molecule of enzyme (1)), causes almost complete loss of enzymatic activity (see Fig. 1). This and the fact that at lower CRDP/enzyme ratios, the extent of inhibition achieved at the time endpoint is proportional to CRDP concentration suggested that the inhibitor binds stoichiometrically with respect to active sites. The presence of divalent magnesium ion, an essential activator (4) in the carboxylase-catalyzed reaction, is also required during the preliminary incubation of enzyme with CRDP for maximal inhibi-

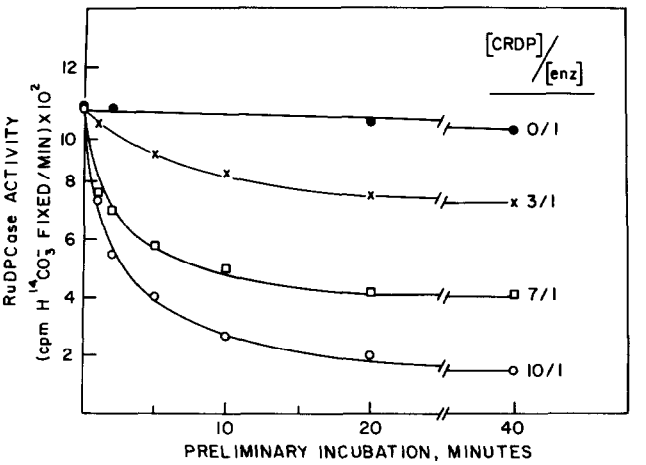


Fig. 1. Dependence of the loss of carboxylase activity on CRDP concentration during preliminary incubation. Homogeneous ribulose diphosphate carboxylase (1.8 x 10^{-8}M) was incubated at 30° in a reaction mixture containing 0.2 M Tris·Cl buffer, pH 7.8, 10 mM $MgCl_2$, 0.05 M [^{14}C] $KHCO_3$ (1.2 x 10^5 c.p.m. per μmole), 0.06 mM EDTA, and 6 mM glutathione in the absence or presence of 5.4 x 10^{-8}M, 1.3 x 10^{-7}M, or 1.8 x 10^{-7}M CRDP. After preliminary incubation for varying periods of time, 0.9 ml aliquots were withdrawn, mixed with 0.1 ml of 7 mM ribulose diphosphate, and the rate of ribulose diphosphate carboxylation determined during a 10 min assay.

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tion (Fig 2). On the other hand, carbon dioxide, the carboxylating substrate species (7), is not required for inhibition by CRDP (results not shown). These results are consistent with our earlier observation (1) that tight binding of CRDP to the carboxylase is observed only in the presence of divalent metal ions, eg., Mg^{2+} or Mn^{2+}, and that the complexes formed contain equimolar amounts of inhibitor and divalent metal ion when isolated by gel filtration.

It is interesting that inhibition by CRDP is a relatively slow process requiring 20-40 min to reach completion at the concentrations of inhibitor employed (see Fig. 1). Under these conditions, not only the extent, but also the rate of inhibition is dependent upon CRDP concentration. Direct binding experiments

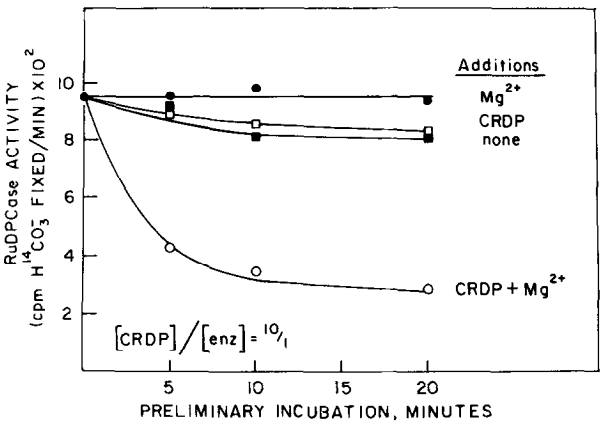

Fig. 2. Dependence of inhibition by CRDP upon the presence of Mg^{2+} during preliminary incubation. The preliminary incubation and carboxylase assays were carried out as described in Fig. 1, but with the variable additions indicated in the Figure. The concentrations of ribulose diphosphate carboxylase and CRDP were 1.8×10^{-8}M and 1.8×10^{-7}M, respectively.

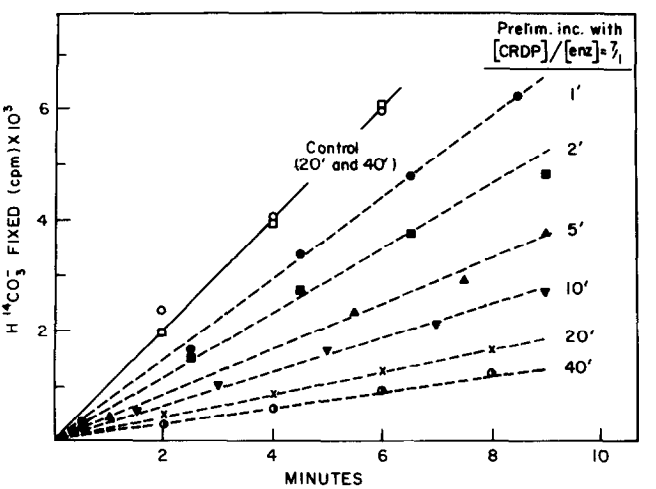

Fig. 3. Kinetics of ribulose diphosphate carboxylation after varying periods of preliminary incubation of carboxylase with CRDP. Preliminary incubation of 1.8×10^{-8}M carboxylase in the absence (Control) or presence of 1.3×10^{-7}M CRDP was carried out as described in Fig. 1; at 1,2,5,10,20 and 40 min, aliquots were taken for carboxylase assays as in Fig. 1.

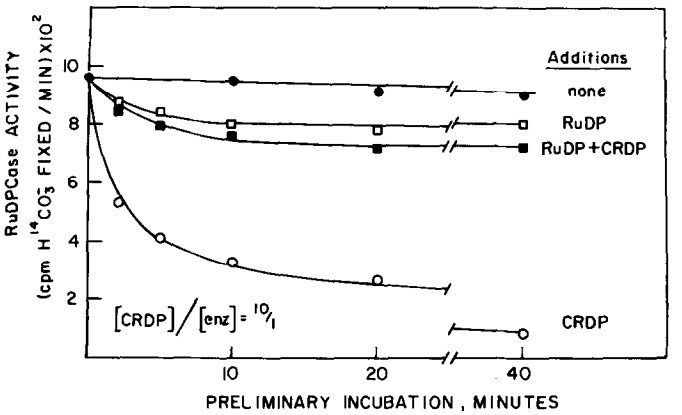

Fig. 4. Protection of the carboxylase by ribulose diphosphate from inhibition by CRDP. Preliminary incubation of the carboxylase (1.8×10^{-8}M) was conducted as described in Fig. 1 except for the variable additions indicated (0.7 mM D-ribulose diphosphate and 1.8×10^{-7}M CRDP) and the omission of [^{14}C] $KHCO_3$. After varying periods of preliminary incubation, aliquots were withdrawn and carboxylase assays initiated by the addition of [^{14}C] $KHCO_3$ (final concentration, 0.05 M) and ribulose diphosphate (only when not present in the preliminary reaction mixture).

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(1) show that CRDP binding to the carboxylase exhibits a similar time dependence. Once CRDP is bound to the enzyme under the conditions outlined in Fig. 1, its release is either extremely slow or does not occur at all. As illustrated in Fig. 3, enzyme withdrawn for assay at any point during the preliminary incubation with inhibitor yielded reduced, but linear, carboxylation rates despite the presence of a high concentration (7×10^{-4}M) of ribulose diphosphate in the assay. This is particularly significant since CRDP and ribulose diphosphate appear to compete for a common binding site, apparently the ribulose diphosphate substrate site. A common binding site is supported by two lines of evidence. First, the presence of ribulose diphosphate during preliminary incubation of the carboxylase with CRDP effectively prevents inhibition (see Fig. 4). Furthermore, the interaction of either ribulose diphosphate or CRDP with the carboxylase produces qualitatively identical

difference spectra in the ultraviolet region. As shown in Fig. 5, both spectra exhibit sharp spikes at 288 nm, as well as minima at 296 nm and 285 nm, and a broad maximum at approximately 265 nm. The height of the 288 nm peak can be titrated to a maximum with ribulose diphosphate or CRDP; at a concentration ratio of CRDP/enzyme of approximately 10, a condition which produces nearly total inhibition of the carboxylase (Fig. 1), the 288 nm peak reaches a maximum. The K_D ($\sim 10^{-8}$M) for the dissociation of CRDP from the ternary complex, calculated from difference spectrum titration data, is identical to that determined by titrating catalytic activity with inhibitor (Fig. 1).

Despite the fact that CRDP binds tightly to the carboxylase at low (4°), as well as, at higher (30°) temeratures (see Table I), the formation of an enzyme·Mg·CRDP complex which is catalytically inactive occurs only upon exposure of the complex to the higher temerature. Hence, the carboxylase-inhibitor complex, prepared at 4° and having 7-8 moles of CRDP bound per mole of enzyme (Experiment I, Table I), is fully active in the standard assay[1]. Merely warming the complex to 30° for a brief period in the absence of ribulose diphosphate, promotes its transition to the inhibited state (Experiments II A and B, Table I), presumably through a temperature-dependent conformation change. Warming the carboxylase in the presence or absence of Mg^{2+} has no detectable effect on catalytic activity. Thus, it appears that inhibition by CRDP can be resolved kinetically into two steps (shown below).

$$\text{Enz} + Mg^{2+} + \text{CRDP} \underset{4°}{\rightleftharpoons} \text{Enz}\cdot Mg^{2+}\cdot\text{CRDP} \xrightarrow[30°]{} (\text{Enz}\cdot Mg^{2+}\cdot\text{CRDP})'$$

[1] In direct kinetic experiments as in Fig. 1, preliminary incubation of the enzyme with CRDP and Mg^{2+}, but at 0°, results in no inhibition in the subsequent assay at 30°.

Table I

Factors affecting inhibition by and binding of CRDP

In Experiment I, 14.8 mg of ribulose diphosphate carboxylase were incubated at 4° for 3 hours in 1 ml containing 0.1 M Tris·Cl buffer, pH 8 at 4°, 0.5 mM [carboxy-^{14}C] CRDP (1.5×10^7 d.p.m. per μmole), and 2 mM $MgSO_4$. The mixture was applied to a 0.9 x 41 cm column of Sephadex G-75 and eluted at 4° with 0.1 M Tris·Cl, pH 8.0, containing 0.1 mM EDTA. After pooling the carboxylase-containing fractions, protein concentration, carboxylase activity, and CRDP bound were determined. Controls without [^{14}C] CRDP in the preliminary incubation mixture were processed in an identical manner. In Experiments II, III, and IV, gel-filtered carboxylase from Experiment I containing 7-8 moles of CRDP per mole of enzyme was subjected to preliminary incubation at 30° with 0.2M Tris·Cl buffer, pH 7.8, containing either: 1) Experiment II - 50 mM $KHCO_3$, 10 mM $MgCl_2$, 6 mM GSH, and 0.1 mM EDTA; 2) Experiment III - 50 mM $KHCO_3$, 0.7 mM ribulose diphosphate, 1.0 mM EDTA and 6 mM GSH; or 3) Experiment IV - 0.5% sodium dodecylsulfate (SDS), 0.1 mM EDTA, and 5 mM 2-mercaptoethanol. Following preliminary incubation, the reaction mixtures were gel-filtered and the eluates analyzed as in Experiment I except that in Experiment IV the eluting buffer also contained 0.5% SDS.

Experiment	Preliminary incubation conditions	Enzyme after gel filtration	
		moles CRDP / mole enzyme	relative specific activity*
			%
I.	180 min at 4° with [^{14}C] CRDP + Mg^{2+}	7-8	94
II.	gel-filtered enz from I;		
(A)	10 min at 30° with Mg^{2+}	7	16
(B)	30 min at 30° with Mg^{2+}	7	11
III.	gel filtered enz from I; 10 min at 30° with RuDP + 1 mM EDTA	2	91
IV.	gel-filtered enz from I; 10 min at 30° with 0.5% SDS	0	-

*activity relative to those of controls treated in an identical manner, but with CRDP omitted in Experiment I.

At 4° an enzyme·Mg·CRDP ternary complex is formed which can be reversed, whereas, at 30° this ternary complex can undergo a further, but irreversible change which locks the enzyme in an inhibited state. Unlike ribulose diphosphate and CO_2 which have

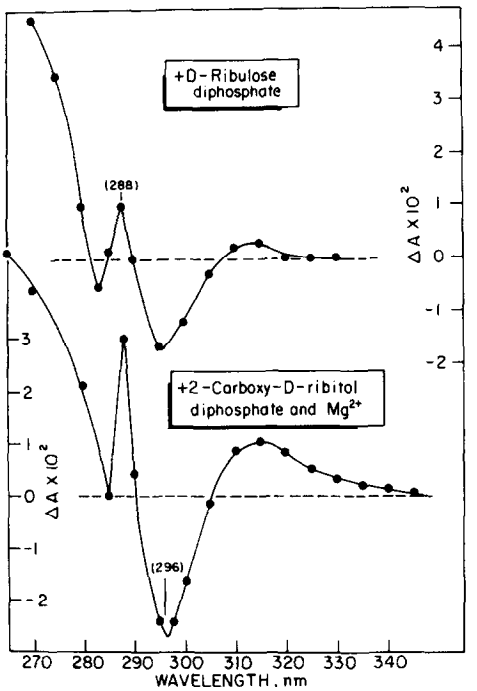

Fig. 5. Difference spectra of ribulose diphosphate carboxylase promoted by D-ribulose-1,5-diphosphate and CRDP. The difference spectrum (1cm light path, 3 ml total volume) of 3 mg of homogeneous carboxylase in 0.25 M Tris·Cl buffer, pH 7.8, 0.1 mM EDTA, 5 mM 2-mercaptoethanol and 1 mM D-ribulose-1, 5-diphosphate versus the same mixture minus ribulose diphosphate was recorded at 30° with a Cary model 15 spectrophotometer. The effect of Mg^{2+} in the presence of ribulose diphosphate could not be tested, since the presence of traces of HCO_3^-, which contaminate the reaction mixture despite the use of "CO_2-free" reagents, allows the enzymatic reaction to proceed. The difference spectrum promoted by 1 mM CRDP was determined as described above except that 10 mM $MgCl_2$ was present in both the experimental and reference cuvets.

easy access to the active site, CRDP which has structural elements of both substrates and has an additional negative charge would be expected to encounter greater difficulty gaining access to the site. However, once this kinetic barrier is surmounted, a conformational "tightening" of the site to accomodate all of its contacts with the inhibitor, perhaps as occurs in the transition state, would markedly strengthen its binding and prevent release. The fact that the Mg^{2+}-dependent binding of CRDP to the carboxylase

under these conditions induces a characteristic spectral change (Fig. 5) supports the view that a conformational change accompanies the formation of the inactive ternary complex. It is clear (Experiments III and IV, Table I) that CRDP is not bound covalently to the carboxylase since its partial removal without activity loss occurs upon incubation with EDTA and ribulose diphosphate and its total release with inactivation is effected with sodium dodecyl sulfate.

The results presented in this communication strongly support Calvin's proposal (2) that a 2-carboxy-3-keto-D-ribitol-1,5-diphosphate intermediate (Structure II) is involved in the carboxylase-catalyzed reaction. Work on the chemical synthesis of the proposed intermediate is currently in progress in our laboratory.

ACKNOWLEDGEMENTS

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