References
Bock, A., and Neidhart, F. C. (1966a), *J. Bacteriol. 92*, 464-469.
Bock, A., and Neidhart, F. C. (1966b), *J. Bacteriol. 92*, 470-476.
de Riel, K. (1976), Ph.D. Thesis, Harvard University.
de Riel, K., and Paulus, H. (1978a), *Biochemistry 17* (first paper in a series of three in this issue).
de Riel, K., and Paulus, H. (1978b), *Biochemistry 17* (second paper in a series of three in this issue).
Dunne, C. P., and Wood, W. A. (1975), *Curr. Top. Cell. Regul. 9*, 65-101.
Frieden, C. (1970), *J. Biol. Chem. 245*, 5788-5799.
Lubin, M., and Ennis, H. L. (1964), *Biochim. Biophys. Acta 80*, 614-631.
Thorner, J. W., and Paulus, H. (1973), *J. Biol. Chem. 248*, 3922-3932.
Zwaig, N., and Lin, E. C. C. (1966), *Science 153*, 755-757.

Effects of Mg^{2+} and Substrates on the Conformation of Ribulose-1,5-bisphosphate Carboxylase†

Alice E. Grebanier, Deborah Champagne, and Harry Roy*

ABSTRACT: Evidence for conformational changes in pea and spinach ribulose-1,5-bisphosphate carboxylase, caused by interaction of the enzyme with ribulose 1,5-bisphophate (RuP_2) or with $MgCl_2$ and $NaHCO_3$, was obtained by measurements of chemical cross-linking and circular dichroism. Samples of the enzyme were incubated with RuP_2 or with $NaHCO_3$ and $MgCl_2$ and then cross-linked with tetranitromethane $C(NO_2)_4$ or dimethyl suberimidate. Cross-linked subunits were detected by dodecyl sulfate-polyacrylamide gel electrophoresis. The formation of most cross-links between subunits with either $C(NO_2)_4$ or dimethyl suberimidate was enhanced by pretreatment with $MgCl_2$ and $NaHCO_3$ and inhibited by pretreatment with RuP_2. A protein species with a slightly faster electrophoretic mobility than the large subunit was formed by treatment with $C(NO_2)_4$. This species is derived from the large subunit, possibly through an intrachain cross-link. The formation of this species was markedly stimulated by pretreatment with $MgCl_2$ and $NaHCO_3$ and almost completely inhibited by pretreatment with RuP_2. The carboxylating activity of the enzyme was inhibited substantially by pretreatment with $C(NO_2)_4$ and slightly by pretreatment with dimethyl suberimidate. Preincubation with $NaHCO_3$ and $MgCl_2$ increased the extent of inactivation by either cross-linking reagent. Preincubation with RuP_2 protected the enzyme activity. The circular dichroism spectrum of the enzyme was altered in magnitude by the addition of $MgCl_2$ and $NaHCO_3$. The greatest shift was at 275 nm. Treatment of RuP_2 carboxylase with $MgCl_2$ and $NaHCO_3$ activates its enzymatic activity. These measurements of chemical cross-linking and circular dichroism indicate that a conformation change occurs in the enzyme upon activation.

Ribulose-1,5-bisphosphate carboxylase [3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1.1.39] catalyzes the carbon dioxide fixation reaction of the photosynthetic carbon reduction cycle and is also possibly involved in glycolate production. The purified enzyme can form two molecules of 3-phosphoglycerate from CO_2 and ribulose 1,5-bisphosphate (RuP_2)[1] (carboxylase activity) or one molecule of phosphoglycolate and one of 3-phosphoglycerate from O_2 and RuP_2 (oxygenase activity). Both reactions of the enzyme are activated by exposure of the protein to $NaHCO_3$ and $MgCl_2$ (Pon et al., 1963; Chu and Bassham, 1973; Lorimer et al., 1976; Badger and Lorimer, 1976; Laing and Christeller, 1976). RuP_2 can bind to the enzyme and inhibit the interconversion of inactive and active forms (Laing and Christeller, 1976).

Evidence that $MgCl_2$, $NaHCO_3$, and RuP_2 cause conformational changes in the enzyme has come from studies using difference spectrophotometry (Rabin and Trown, 1964; Kwok and Wildman, 1974), fluorometry of added probes (Wildner, 1976; Vater et al., 1977), and measurement of inactivation of the enzyme by chemical modifiers (Schloss and Hartmann, 1977).

In eukaryotic plants, the enzyme is comprised of two types of subunit, L (M_r 55 000) and S (M_r 12 000-14 000) (Kung, 1976). Earlier work in this laboratory has shown that it is possible to probe the association of these subunits through the use of chemical cross-linking reagents (Roy et al., 1978). The present study shows that this kind of structural analysis can be used to detect conformational changes in the enzyme caused by binding of substrate molecules, providing information of a different sort than the probes mentioned above yield. Additionally, conformational changes were detected by circular dichroism. The circular dichroism of RuP_2 carboxylase is altered by $MgCl_2$ and $NaHCO_3$.

Experimental Procedures

Enzyme Isolation. RuP_2 carboxylase was purified from homogenates of pea seedling shoots (*Pisum sativum*, variety Progress no. 9, Agway, Buffalo, N.Y.) as described previously (Roy et al., 1978), except for the final chromatographic step. The enzyme was loaded onto a 2.5 × 10 cm DEAE-Sephadex A-50 column which had been equilibrated with 0.05 M Tris-HCl (pH 7.5), 0.05 M NaCl. A 300-mL linear gradient of 0.05 to 0.30 M NaCl in the same buffer was used to elute the en-

† From the Department of Biology, Rensselaer Polytechnic Institute, Troy, New York 12181. *Received July 5, 1978.* Supported by National Institutes of Health Grant GM 23353 to H.R.

[1] Abbreviations used: RuP_2, ribulose 1,5-bisphosphate; L, large subunit of RuP_2 carboxylase; S, small subunit of RuP_2 carboxylase; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

0006-2960/78/0417-5150$01.00/0

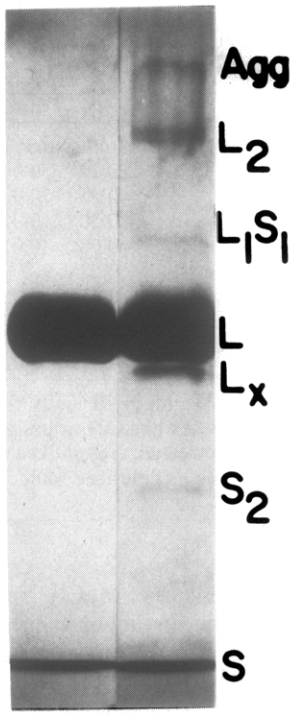

FIGURE 1: Analysis of RuP_2 carboxylase by dodecyl sulfate–polyacrylamide gel electrophoresis. Samples were prepared as described under Experimental Procedures: (left) RuP_2 carboxylase not treated with cross-linking reagents; (right) RuP_2 carboxylase cross-linked with $C(NO_2)_4$.

zyme, which was then stored as a precipitate in 50% saturated $(NH_4)_2SO_4$ at 2 °C. Immediately before use, the enzyme was desalted on a Sephadex G-25 column (1 × 14 cm) equilibrated with 0.05 M Tris-HCl (pH 8.0) or 0.05 M triethanolamine hydrochloride (pH 8.0). The enzyme was analyzed by dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli, 1970) for the presence of polypeptides other than L or S and was judged to be greater than 99% pure.

Assay of Carboxylation of RuP_2. To test activation, 1 mg/mL solutions of RuP_2 carboxylase in 0.05 M Tris-HCl (pH 8.0) or 0.05 M triethanolamine hydrochloride (pH 8.0) were either pretreated for 5 min at 20 °C with 0.01 M $NaHCO_3$ and 0.02 M $MgCl_2$ or they were not pretreated. Then the carboxylation reaction was started by the addition of 0.01 mL of the enzyme to 0.19 mL of 0.05 M Tris-HCl (pH 8.0), 0.01 M $MgCl_2$, 0.0005 M RuP_2, 0.0105 M $NaHCO_3$, 5 μCi of $NaH^{14}CO_3$ and stopped 1 min later by the addition of 0.05 mL of 4 N HCl. The samples were evaporated to dryness, the residue was dissolved in 0.3 mL of H_2O, and 4 mL of scintillation fluid was added [33.3% Triton X-100/61.2% toluene/5.6% Liquifluor (New England Nuclear) (v/v)].

For assay of the activity of cross-linked samples, cross-linking reagents and metabolites were first removed by desalting the enzyme on a Sephadex G-25 column (1 × 14 cm) which had been equilibrated with 0.05 M Tris-HCl (pH 8.0). Ten micrograms of the enzyme was activated by a 5-min preincubation at 20 °C in 0.19 mL of 0.05 M Tris-HCl (pH 8.0), 0.0105 M $NaHCO_3$ containing 2 μCi of $NaH^{14}CO_3$, 0.021 M $MgCl_2$. The reaction was started by the addition of 0.01 mL of 0.01 M RuP_2 and stopped 10 min later by the addition of 0.05 mL of 4 N HCl. Samples were prepared for scintillation counting as described above.

Cross-linking Reactions. Before cross-linking reagents were added, samples of the enzyme were pretreated with and without substrates. A 1 mg/mL solution of RuP_2 carboxylase was incubated with 5×10^{-4} M RuP_2 or with 0.01 M $NaHCO_3$ and 0.02 M $MgCl_2$ for 5 min at 20 °C. For cross-linking with tetranitromethane, the buffer was 0.05 M Tris-HCl (pH 8.0). For cross-linking with dimethyl suberimidate, the buffer was 0.05 M triethanolamine hydrochloride (pH 8.0). In some experiments, RuP_2 was added along with the cross-linking reagent to samples which had been pretreated with $NaHCO_3$ and $MgCl_2$. Other samples had the reverse order of additions, but the same final composition.

Stock solutions of 0.028 M $C(NO_2)_4$ in 95% ethanol and 0.11 M dimethyl suberimidate in 0.5 M triethanolamine hydrochloride (pH 8.5) were prepared immediately before use. Pretreated enzyme was incubated with 0.0005 M $C(NO_2)_4$ for 10 min at 20 °C or with 0.011 M dimethyl suberimidate for 30 min in an ice bath.

For analysis by electrophoresis, samples were heated at 90 °C for 2 min with a dodecyl sulfate containing solution (Laemmli, 1970). Then, 45-μg samples were loaded into the wells of a dodecyl sulfate–polyacrylamide slab gel for electrophoresis. The running gel (9 × 14 × 0.1 cm) was 7.5% and the stacking gel (1.5 × 14 × 0.1 cm) was 5% acrylamide in a discontinuous buffer system (Laemmli, 1970). After staining with 0.2% Coomassie blue R, 43% ethanol, and 10% acetic acid and destaining with 9.5% ethanol and 10% acetic acid, gels were scanned at 590 nm.

One-Dimensional Mapping of Proteolytic Fragments. Protein bands on a polyacrylamide gel were located by light staining, excised with a razor blade, placed in the wells of a second gel, overlayered with 0.3 μg of papain or *Staphlococcus aureus* protease, and electrophoresed, all as described previously (Cleveland et al., 1977). Samples containing only the proteolytic enzyme were included for comparison. The running gel was a linear 12 to 16% polyacrylamide gradient, and the stacking gel was 5% acrylamide, in a discontinuous buffer system (Laemmli, 1970).

Circular Dichroism Measurements. Circular dichroism spectra in the region 320 to 250 nm were determined on a Durrum-JASCO J5 optical rotatory dispersion spectrometer equipped with a circular dichroism attachment (Sproul Scientific Co.). Samples were clarified by table-top centrifugation in a swinging bucket in conical tubes. This had no effect on the general pattern of the signals or on changes in magnitude caused by $MgCl_2$ and $NaHCO_3$. The same effects were observed whether these salts were added by mixing or by dialysis.

Reagents. RuP_2, $C(NO_2)_4$, and Tris base were obtained from Sigma, dimethyl suberimidate was from Pierce, triethanolamine was from Fisher, and $NaH^{14}CO_3$ (50 Ci/mol) was from New England Nuclear. All inorganic salts were analytical grade.

Results

RuP_2 carboxylase has been reported to be activated by $NaHCO_3$ and $MgCl_2$. We verified that, in either of the buffer systems used in the cross-linking experiments, treatment of the enzyme with 0.01 M $NaHCO_3$ and 0.02 M $MgCl_2$ enhanced the carboxylation rate to a value of 110 nmol of CO_2 (mg of protein)$^{-1}$ min^{-1}. Without activation, the rate was 12 nmol of CO_2 (mg of protein)$^{-1}$ min^{-1}.

Cross-linked Polypeptides of RuP_2 Carboxylase. Treatment of RuP_2 carboxylase with $C(NO_2)_4$ causes increases in the amounts of several protein bands, which are detected when the enzyme subunits are separated by dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 1). Because of the short cross-linking times used, the cross-linked species were a small

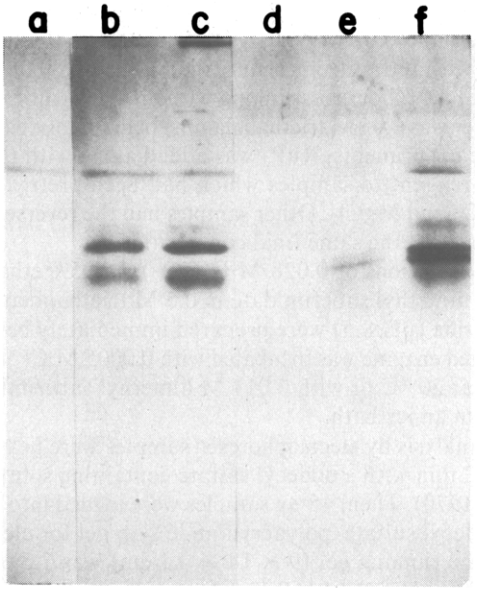

FIGURE 2: One-dimensional maps of proteolytic fragments of L and L_x. Protein bands L and L_x were excised from dodecyl sulfate–polyacrylamide gels of untreated or cross-linked RuP_2 carboxylase and digested with 0.3 μg of papain or *S. aureus* protease on a second dodecyl sulfate–polyacrylamide gel as described under Experimental Procedures: (a) *S. aureus* protease alone; (b) L and *S. aureus* protease; (c) L_x and *S. aureus* protease; (d) papain alone; (e) L and papain; (f) L_x and papain.

fraction of the total protein, and heavy loading of the gels was required to permit their detection. Consequently, the staining intensity of L and S was so high that concomitant decreases in the amounts of monomeric L and S upon cross-linking could not be measured. Some of the cross-linked polypeptides have been identified as a dimer of small subunit (S_2), an aggregate of large and small subunits (L_1S_1), and a dimer of large subunit (L_2) (Roy et al., 1978). Also, there are higher order aggregates, which migrate more slowly during electrophoresis (Agg), and a prominent, sharp protein band (L_x) which migrates just faster than the large subunit. The latter did not show up so clearly in earlier work in which the enzyme was not pretreated with $MgCl_2$ and $NaHCO_3$ (Roy et al., 1978). Its prominence in Figure 1 is due to the activation of the enzyme before cross-linking (see below).

Identity of L_x. One-dimensional mapping of proteolytic fragments was used to determine the origin of L_x. Slices of polyacrylamide gel containing L_x from a cross-linked sample or L from an untreated one were placed in wells of a slab gel, overlayered with proteolytic enzymes, and electrophoresed. This procedure generates discrete fragments, the electrophoretic mobilities of which are characteristic for the protease used and the protein digested (Cleveland et al., 1977). The maps of papain digests and of *S. aureus* protease digests of L_x were identical to those of digests of L (Figure 2). Thus, we concluded that L_x is derived from L. The one-dimensional maps do not show the nature of the difference between L and L_x.

Relationship of the Cross-linking Pattern to Activity. The extent of cross-linking by $C(NO_2)_4$ was dependent on prior treatment with $MgCl_2$ and substrates (Table I). The formation of L_x was more sensitive to such pretreatment than was the formation of any other cross-linked material. Without any pretreatment, L_x formation by $C(NO_2)_4$ was twofold above the background level. When the enzyme was activated by $MgCl_2$ and $NaHCO_3$, however, L_x formation by $C(NO_2)_4$ was sixfold above background. $NaHCO_3$ alone had little effect on L_x formation. RuP_2 prevented any increase in L_x over the background amount.

TABLE I: Cross-linking of Pea RuP_2 Carboxylase with $C(NO_2)_4$.[a]

pretreatment	cross-linked species			
	L_2	L_1S_1	L_x	S_2
	cross-linked with $C(NO_2)_4$			
none	0.22	0.04	0.19	0.07
$NaHCO_3$, $MgCl_2$	0.25	0.03	0.62	0.10
$NaHCO_3$	0.20	0.03	0.21	0.07
RuP_2	0.08	0.02	0.09	0.04
$NaHCO_3$, $MgCl_2$, then RuP_2	0.22	0.03	0.58	0.08
RuP_2, then $NaHCO_3$, $MgCl_2$	0.21	0.05	0.16	0.06
	not cross-linked			
none	0.01	0.02	0.10	0.03

[a] Values given are peak heights in OD units, from scans of stained gels at 590 nm. L and S peaks had OD values greater than 3 in all samples. Samples were pretreated, cross-linked with $C(NO_2)_4$, and analyzed by electrophoresis as described under Experimental Procedures.

The simultaneous presence of $MgCl_2$, $NaHCO_3$, and RuP_2 gave different results, depending on their order of addition. When RuP_2 was supplied in the preincubation and $MgCl_2$ and $NaHCO_3$ were added with $C(NO_2)_4$, L_x formation was only slightly above the background levels obtained with RuP_2 alone. If RuP_2 was added with $C(NO_2)_4$ to enzyme preincubated with $MgCl_2$ and $NaHCO_3$, L_x formation by $C(NO_2)_4$ was still sixfold above background. It should be noted that when the enzyme was activated with $MgCl_2$ and $NaHCO_3$, the RuP_2 was broken down to 3-phosphoglycerate during the first few minutes of the $C(NO_2)_4$ treatment (data not shown). This possibly accounts for the lack of an RuP_2 effect on L_x formation in this sample. When the enzyme was treated with RuP_2 before the addition of $MgCl_2$ and $NaHCO_3$, the rate of carboxylation was lower, so that RuP_2 was present during the entire time of $C(NO_2)_4$ treatment.

Formation of L_2 and S_2 was higher when the enzyme had been activated; however, the effects on L_2 and S_2 were less dramatic than those on L_x formation. In the experiment cited in Table I, L_2 and S_2 were only slightly stimulated by activation of the enzyme [as compared to their formation by $C(NO_2)_4$ without any pretreatment]. In other experiments, up to a twofold increase in the amount of these species, caused by preincubation with $NaHCO_3$ and $MgCl_2$, was seen. RuP_2 had a more consistent effect, suppressing formation of L_2 and S_2 by at least 60% in all experiments; however, RuP_2 was never observed to block all L_2 and S_2 formation. The effects of pretreatment with RuP_2 on L_2 and S_2 formation were largely overcome by $MgCl_2$ and $NaHCO_3$ added with $C(NO_2)_4$. This is in marked contrast to the effect of RuP_2 on L_x formation, which is much more resistant to reversal by $MgCl_2$ and $NaHCO_3$.

L_1S_1 was formed only in minor amounts in the pea enzyme, so that it was difficult to assess the effects of pretreatment on its formation.

The effects of the pretreatment of pea RuP_2 carboxylase on the formation of oligomers with dimethyl suberimidate are similar to the effects seen with $C(NO_2)_4$ (Table II). The products of cross-linking show similar mobilities to those of cross-linking by $C(NO_2)_4$ (Roy et al., 1978); however, dimethyl suberimidate does not cause the appearance of L_x, and the amount of S_2 formed in these experiments was too low to permit its quantitation. L_1S_1 formation by dimethyl suberimidate was greater than with $C(NO_2)_4$, and the amount of L_1S_1 formed was clearly increased by treatment of the enzyme with $NaHCO_3$ and $MgCl_2$ prior to the addition of dimethyl

TABLE II: Cross-linking of Pea RuP_2 Carboxylase with Dimethyl Suberimidate.[a]

pretreatment	cross-linked species L_2	cross-linked species L_1S_1
	cross-linked with dimethyl suberimidate	
none	0.19	0.06
$NaHCO_3$, $MgCl_2$	0.26	0.09
$NaHCO_3$	0.17	0.04
RuP_2	0.10	0.04
	not cross-linked	
none	0.00	0.02

[a] Values given are peak heights in OD units, from scans of stained gels at 590 nm. L and S peaks had OD values greater than 3 in all samples. L_x and S_2 were not detectable in any of these samples. Samples were pretreated, cross-linked with dimethyl suberimidate, and analyzed by electrophoresis as described under Experimental Procedures.

TABLE III: Cross-linking of Spinach RuP_2 Carboxylase.[a]

pretreatment	cross-linked species L_2	L_1S_1	L_x	S_2
	cross-linked with $C(NO_2)_4$			
none	0.51	1.26	0.13	0.12
$NaHCO_3$, $MgCl_2$	0.63	0.92	0.21	0.14
RuP_2	0.30	1.22	0.02	0.03
	cross-linked with dimethyl suberimidate			
none	0.24	0.16	0.02	0.06
$NaHCO_3$, $MgCl_2$	0.46	0.24	0.01	0.10
RuP_2	0.15	0.12	0.02	0.05
	not cross-linked			
none	0.01	0.02	0.02	0.01

[a] Values given are peak heights in OD units, from scans of stained gels at 590 nm. L and S peaks had OD values greater than 3. Samples were pretreated, cross-linked with $C(NO_2)_4$ or dimethyl suberimidate and analyzed by electrophoresis as described under Experimental Procedures.

suberimidate. A similar pattern of effects on L_2 formation was also seen.

In order to minimize the effects of alterations in the enzyme's structure caused by interactions with the cross-linking reagents, the time of treatment with these reagents was usually kept to the minimum needed to give a measurable degree of cross-linking. However, even with treatment of up to an hour with either $C(NO_2)_4$ or dimethyl suberimidate, differential extents of cross-linking were seen, depending on pretreatment of the enzyme with its substrates, similar in nature to those described above.

Cross-linking of Spinach RuP_2 Carboxylase. To determine whether the effects of preincubation on cross-linking were specific for the pea enzyme a similar set of experiments was performed using spinach RuP_2 carboxylase. The relative abundance of particular cross-linked proteins was different for the enzymes from the two plants (compare Tables I–III). The most striking difference was in the amount of L_1S_1 formed after treatment with $C(NO_2)_4$. L_1S_1 is the major product with the spinach enzyme, but only marginally detectable with the pea enzyme.

Interestingly, the extent of L_1S_1 formation by $C(NO_2)_4$ is depressed by treatment with $NaHCO_3$ and $MgCl_2$, while L_1S_1 formation by dimethyl suberimidate is enhanced by the same treatment.

TABLE IV: Inactivation of RuP_2 Carboxylase by $C(NO_2)_4$ and Dimethyl Suberimidate.[a]

pretreatment	cross-linking	nmol of CO_2 (mg of protein)$^{-1}$ min^{-1} (±SD) $C(NO_2)_4$	nmol of CO_2 (mg of protein)$^{-1}$ min^{-1} (±SD) dimethyl suberimidate
none	−	84 (±5.7)	77 (±2.4)
none	+	42 (±5.7)	63 (±0.5)
$NaHCO_3$, $MgCl_2$	+	2.6 (±0.1)	52 (±4.4)
$NaHCO_3$	+	41 (±1.7)	60 (±0.5)
RuP_2	+	77 (±5.2)	70 (±3.0)

[a] Pretreated samples were cross-linked and then desalted, and the carboxylation activities were measured, all as described under Experimental Procedures. SD values are for triplicate measurements of single samples of protein.

In all other respects, the results were essentially the same as for pea RuP_2 carboxylase. Generally, pretreatment with $NaHCO_3$ and $MgCl_2$ enhanced cross-linking, while pretreatment with RuP_2 was inhibitory.

Sensitivity to Inhibition of Activity by Cross-linkers. The sensitivity of the enzyme to $NaHCO_3$, $MgCl_2$, and RuP_2 could also be monitored by assessing their effects on the ability of the cross-linkers to inactivate the carboxylation reaction (Table IV). Pea RuP_2 carboxylase was pretreated with $NaHCO_3$ and $MgCl_2$ or with RuP_2 and then treated with the cross-linkers exactly as described for the electrophoretic experiments shown in Tables I and II. In this case, however, the cross-linked samples were fractionated on a Sephadex column to separate the enzyme from substrates and cross-linkers. The desalted, cross-linked enzyme was then incubated in $MgCl_2$ and $NaHCO_3$ to activate it, and carboxylation of RuP_2 was measured. Since no $C(NO_2)_4$ or dimethyl suberimidate was present in the reaction mixture, any effects seen are due to irreversible reactions of the cross-linkers with the enzyme. When the enzyme was activated by $MgCl_2$ and $NaHCO_3$ and then treated with $C(NO_2)_4$, there was a 97% inhibition of the subsequently measured carboxylation activity. If $MgCl_2$ and $NaHCO_3$ were omitted, the inhibition was only 50%. When RuP_2 was present during cross-linking, the inhibition of carboxylation activity by the cross-linker was only 9%. With dimethyl suberimidate as the cross-linker, the inhibition was less than with $C(NO_2)_4$, but the same pattern of potentiation of inactivation by $MgCl_2$ and $NaHCO_3$ and protection by RuP_2 was observed.

Circular Dichroism Measurements. Based on the results of the electrophoretic and enzymatic analysis of the cross-linked enzyme, it seemed desirable to add circular dichroism measurements of conformational changes to monitor effects of $MgCl_2$ and substrates on the enzyme. The addition of $MgCl_2$ and $NaHCO_3$ causes a change in the circular dichroism spectrum (Figure 3). The most noticeable feature is a general shift toward the base line across the near-ultraviolet region; there is also a more marked shift in this direction at about 275 nm. The largest negative peak occurs at 287 nm and is used as a measure of the change in Figure 3. Upon removal of $MgCl_2$ and $NaHCO_3$, this signal returns to its original value, within experimental error. Readdition of the salts brings about the positive shift once more. The effect of these salts on RuP_2 carboxylase activity is also reversible (Badger and Lorimer, 1976; Lorimer et al., 1976). A similar pattern, and a similar shift in magnitude of the signals upon the addition of $MgCl_2$ and $NaHCO_3$, was observed with the spinach enzyme (not shown). The circular dichroism peaks at 275, 287, and 294 nm

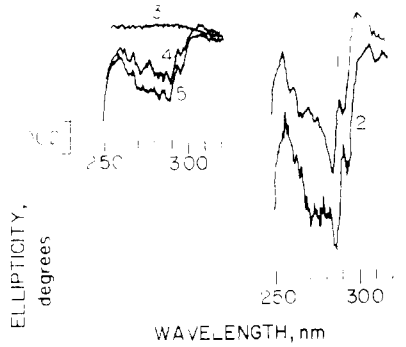

FIGURE 3: Circular dichroism of pea RuP_2 carboxylase: (1) Freshly isolated RuP_2 carboxylase at 3.3×10^{-7} dmol/cm^3 in 0.01 M Tris-HCl (pH 8.0), 0.02 M $MgCl_2$, 0.01 M $NaHCO_3$; molar ellipticity at 287 nm = -2.8×10^4 deg-cm^2/dmol. (2) Same as 1 but without $MgCl_2$ and $NaHCO_3$, 3.8×10^{-7} dmol/cm^3; molar ellipticity at 287 nm = -3.8×10^4 deg-cm^2/dmol. (3) Base line (0.01 M Tris-HCl, pH 8.0); $MgCl_2$ and $NaHCO_3$ had no effect on the base line. (4) RuP_2 carboxylase treated as in 1, reprecipitated with $(NH_4)_2SO_4$, and dialyzed in 0.01 M Tris-HCl (pH 8.0), followed by the addition of 0.02 M $MgCl_2$, 0.01 M $NaHCO_3$, 1.09×10^{-7} dmol/cm^3; molar ellipticity at 287 nm = -2.9 deg-cm^2/dmol. (5) RuP_2 carboxylase treated as in 4, but no $MgCl_2$ and $NaHCO_3$ were added after reprecipitation by ammonium sulfate, 1.05×10^{-7} dmol/cm^3; molar ellipticity at 287 nm = -4.4×10^4 deg-cm^2/dmol. Base line for recording 1 and 2 is at same level as that for recordings 4 and 5.

have not been identified with any particular amino acids so far. RuP_2 itself has a large, negative circular dichroism peak at 283 nm; thus, the effect of RuP_2 on circular dichroism of RuP_2 carboxylase has not been evaluated.

Discussion

Since the enzymatic activity of RuP_2 carboxylase is affected by pretreatment with $NaHCO_3$, $MgCl_2$, and RuP_2, one might reasonably suppose that there are conformational changes in the protein associated with its interactions with these molecules. Physical evidence in support of this idea has been reported by others (Kwok and Wildman, 1974; Rabin and Trown, 1964; Vater et al., 1977; Wildner, 1976). We have obtained evidence for such changes from two kinds of probes of protein structure: chemical cross-linking of subunits and circular dichroism.

The bulk of our evidence for a general conformational change in the enzyme comes from experiments with cross-linking reagents. The extent of cross-linking between subunits of RuP_2 carboxylase by $C(NO_2)_4$ or dimethyl suberimidate depends upon which of the enzyme's substrates are present during a preincubation of the enzyme. If the enzyme has been activated by pretreatment with $NaHCO_3$ and $MgCl_2$, there is enhanced cross-linking. $NaHCO_3$ without $MgCl_2$ has no effect on cross-linking and does not activate the enzyme. $MgCl_2$, without added $NaHCO_3$, caused both activation of the carboxylation reaction and enhancement of cross-linking, though to a lesser extent in either case than when $NaHCO_3$ was also added (data not shown). The latter results are explained by the fact that we did not attempt to maintain CO_2-free solutions, so that CO_2 and HCO_3^-, in equilibrium with the CO_2 in the air, were always present, even without added $NaHCO_3$.

If the enzyme is pretreated with RuP_2, the activity is decreased, and there is diminished cross-linking. It is not certain whether the inactivation is due to RuP_2 itself or to unavoidable contaminants in RuP_2 (McCurry et al., 1978). However, even if RuP_2 is not the active agent, a correlation between the stabilization of the enzyme in an inactive form and a decrease in extent of cross-linking can be made.

The effects of $MgCl_2$, $NaHCO_3$, and RuP_2 on cross-linking could be due either to alterations of the conformation of the enzyme or else to direct interactions between these substances and the cross-linking reagents. Two observations argue against the latter possibility. First, similar patterns of dependence on pretreatment conditions for the appearance of particular cross-linked species were seen with both $C(NO_2)_4$ and dimethyl suberimidate, although the chemical mechanisms for cross-linking with the two reagents are different. $C(NO_2)_4$ cross-links tyrosines (Williams and Lowe, 1966), and dimethyl suberimidate cross-links primary amino groups (Hartmann and Wold, 1966). [$C(NO_2)_4$ can also cause the formation of disulfide bonds between cysteine residues (Sokolovsky et al., 1966), but this kind of cross-linking was not detected in the experiments described here because of the reduction of samples with 2-mercaptoethanol before electrophoresis.] Secondly, there is a differential effect of pretreatment conditions on the appearance of different cross-linked species. The formation of L_x by treatment with $C(NO_2)_4$ is much more sensitive to pretreatment of the enzyme than is formation of L_2 with the same reagent.

Thus, the effects of the enzyme's substrates on the degree of cross-linking are most probably due to effects on the conformation of the enzyme. When the enzyme is activated, its subunits are closer together. Both chemical cross-linkers detect this.

The details of the changes in cross-linking suggest that very specific structural alterations in different parts of the molecule can be monitored in a single sample. For example, the decrease in L_1S_1 formed by $C(NO_2)_4$, caused by activation of the spinach enzyme, may reflect a movement in position of cross-linked residues. $C(NO_2)_4$ is a "zero-length" cross-linker (Martinson and McCarthy, 1975); no element of $C(NO_2)_4$ is present in the cross-linked species (Williams and Lowe, 1966). Thus, the formation of cross-links will be highly sensitive to slight conformational changes. The *decrease* in L_1S_1 formed by $C(NO_2)_4$ upon activation is not necessarily inconsistent with the *increase* in L_1S_1 formed by dimethyl suberimidate under the same conditions, or with the conclusion that, in the activated enzyme, the subunits are closer together.

A similar conclusion was reached for conformational changes in tobacco RuP_2 carboxylase with reference to cold inactivation and heat reactivation. Chollet and Anderson (1977), through sulfhydryl group titration and measurement of 8-anilino-1-naphthalenesulfonate fluorescence, supported the suggestion of Kawashima et al. (1971) that cold inactivation is associated with a partial dissociation of the enzyme subunits. The observations reported here extend this conclusion from thermal effects to the effects of $NaHCO_3$, $MgCl_2$, and RuP_2.

The formation of L_x by $C(NO_2)_4$ is interesting, because its appearance is most highly dependent on prior activation of the enzyme, and most completely inhibited by treatment with RuP_2. L_x formation may reflect a specific alteration of the large subunit conformation associated with the activation process. L_x is present in most of our preparations of RuP_2 carboxylase, even without cross-linking treatment, but only in very minor amounts (less than 1% of the amount of L protein). $C(NO_2)_4$ is the only cross-linking reagent we have tested which enhances L_x formation. The protease digestion data show that L_x is derived from L; however, the nature of the difference between L and L_x has not been determined. One possibility is that L_x is L with an intrachain cross-link which causes a "crimp" in the polypeptide, lowering the molecular volume of its complex with dodecyl sulfate, or increasing its charge, or both. The possibility that L_x is derived by cleavage

of L seems remote.

RuP_2 almost completely suppresses the formation of L_x by $C(NO_2)_4$. We hesitate to attribute the effect to protection of the RuP_2 binding sites of the enzyme, since RuP_2 also decreases cross-linking between adjacent subunits. Obviously, the binding of RuP_2 to the enzyme can have long-range conformational effects.

A second indication of conformational changes in RuP_2 carboxylase is the pattern of sensitivity of the carboxylating activity to inhibition by $C(NO_2)_4$ and dimethyl suberimidate, depending upon prior treatment of the enzyme with $MgCl_2$ and substrates. The extent of cross-linking and the inhibition of enzyme activity both show similar dependencies on metabolites present before the addition of $C(NO_2)_4$ or dimethyl suberimidate. In the case of $C(NO_2)_4$, however, it has been found that inhibition occurs at far lower concentrations than those required for cross-linking (J. Patrizio, unpublished data). Thus, the inhibition may not be due to cross-linking, but perhaps may be due to the nitration of cysteine or tyrosine residues (Mayaudon et al., 1957; Sokolovsky et al., 1969). In the case of dimethyl suberimidate, it is possible that inhibition results from reaction with essential lysyl residues (Norton et al., 1975) rather than from immobilization of subunits upon cross-linking. Even though the exact mechanism of inhibition is unknown, the effects of preincubation with substrates support the idea that the inhibition sites are exposed differently in the active and inactive conformations of RuP_2 carboxylase.

A third indication of conformational changes comes from a circular dichroism study. In the near-ultraviolet region (250 to 320 nm), pea and spinach RuP_2 carboxylase exhibit several discrete circular dichroism signals which are probably due to asymmetry in the microenvironment of aromatic amino acids. All of these signals are affected in a consistent, reversible manner by the addition of $MgCl_2$ and $NaHCO_3$. The possibility that these changes are due to aggregation or some other artifact has been minimized by controls which show that removal of aggregates by centrifugation or substitution of various salts or buffers is without effect. Although we have not identified any of the circular dichroism peaks with particular amino acids, it seems as if a general alteration in structure must be occurring. There are changes in the magnitudes of several peaks. Some of these changes are larger than others. Effects of RuP_2 on the circular dichroism of the enzyme involve corrections for the circular dichroism of RuP_2 and are still being investigated. Nevertheless, these data establish that the enzyme activation is associated with changes detected by a well-recognized probe of tertiary structure.

The evidence presented here confirms and extends the observations of other investigators that a conformational change in RuP_2 carboxylase occurs upon activation of the enzyme. The chemical cross-linking method of monitoring this change shows details of this process which may be less accessible to other methods of analysis.

Acknowledgment

The authors thank Dr. Joseph Mascarenhas for the use of his linear gel transporter, Dr. Herbert Richtol for the use of his circular dichroism spectrometer, and Kris Costa for technical assistance.

References

Badger, M. R., and Lorimer, G. H. (1976), *Arch. Biochem. Biophys. 175,* 723.

Chollet, R., and Anderson, L. L. (1977), *Biochim. Biophys. Acta 482,* 228.

Chu, D. K., and Bassham, J. A. (1973), *Plant Physiol. 52,* 373.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977), *J. Biol. Chem. 252,* 1102.

Hartmann, F. C., and Wold, F. (1966), *J. Am. Chem. Soc. 88,* 3890.

Kawashima, N., Singh, S., and Wildman, S. G. (1971), *Biochem. Biophys. Res. Commun. 42,* 664.

Kung, S.-D. (1976), *Science 191,* 429.

Kwok, S. Y., and Wildman, S. G. (1974), *Arch. Biochem. Biophys. 161,* 354.

Laemmli, U. K. (1970), *Nature (London) 227,* 680.

Laing, W. A., and Christeller, J. T. (1976), *Biochem. J. 159,* 563.

Lorimer, G. H., Badger, M. R., and Andrews, J. T. (1976), *Biochemistry 15,* 529.

Martinson, H. G., and McCarthy, B. J. (1975), *Biochemistry 14,* 1073.

Mayaudon, J., Benson, A. A., and Calvin, M. (1957), *Biochim. Biophys. Acta 23,* 342.

McCurry, S. D., Paech, C., Pierce, J., and Tolbert, N. E. (1978), *Plant Physiol. Suppl. 61,* 99.

Norton, I. L., Welch, M. H., and Hartmann, F. C. (1975), *J. Biol. Chem. 250,* 8062.

Pon, N. G., Rabin, B. R., and Calvin, B. (1963), *Biochem. Z. 338,* 7.

Rabin, B. R., and Trown, P. W. (1964), *Nature (London) 202,* 1290.

Roy, H., Valeri, A., Pope, D. H., Rueckert, L., and Costa, K. A. (1978), *Biochemistry 17,* 665.

Schloss, J. V., and Hartmann, F. C. (1977), *Biochem. Biophys. Res. Commun. 77,* 230.

Sokolovsky, M., Harell, D., and Riordan, J. F. (1969), *Biochemistry 8,* 4740.

Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1966), *Biochemistry 5,* 3582.

Vater, J., Salnikow, J., and Kleinkauf, H. (1977), *Biochem. Biophys. Res. Commun. 74,* 1618.

Wildner, G. F. (1976), *Z. Naturforsch. C 31,* 267.

Williams, J., and Lowe, J. M. (1971), *Biochem. J. 121,* 203.