Inactivation of ribulosebisphosphate carboxylase by modification of arginyl residues with phenylglyoxal

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amino-acid oxidase with propargylglycine, in which both irreversible (Marcotte and Walsh, 1978a) and reversible (Marcotte and Walsh, 1978b) interactions are observed. This work has demonstrated that, when reaction between an enzyme and a substrate analogue results in release of a highly reactive product, it is necessary to characterize the pathway following the enzymatic step to determine if any species formed subsequently interacts with that enzyme or other enzymes in vivo.

References

Abeles, R. H., and Walsh, C. T. (1973), *J. Am. Chem. Soc. 95,* 6124.

Alberici, M., Rodriquez de Lores Arnaiz, G., and DeRobertis, E. (1969), *Biochem. Pharmacol. 18,* 137.

Baldwin, J. E., Haber, S. B., Hoskins, C., and Kruse, L. I. (1977), *J. Org. Chem. 42,* 1239.

Barlow, G. B., and MacLeod, A. J. (1964), *J. Chem. Soc.,* 141.

Bayly, R. C., and Dagley, S. (1969), *Biochem. J. 111,* 303.

Brumby, P., and Massey, V. (1968), *Biochem. Prep. 12,* 29.

Collingsworth, W. L., Chapman, P. J., and Dagley, S. (1973), *J. Bacteriol. 113,* 922.

Cromartie, T., Fisher, J., Kaczrowski, G., Laura, R., Marcotte, P., and Walsh, C. (1974), *J. Chem. Soc., Chem. Commun.,* 597.

Curti, B., Ronchi, S., Branzoli, U., Ferri, G., and Williams, C. H. (1973), *Biochim. Biophys. Acta 327,* 266.

Dagley, S., and Gibson, D. T. (1965) *Biochem. J. 95,* 466.

Horton, R. W. (1978), *Biochem. Pharmacol. 27,* 1471.

Horton, R. W., and Meldrum, B. S. (1973), *Br. J. Pharmacol. 49,* 52.

Horton, R. W., and Meldrum, B. S. (1977), *Br. J. Pharmacol. 61,* 477P.

McFarland, D., and Wainer, A. (1965), *Life Sci. 4,* 1587.

Marcotte, P., and Walsh, C. (1975), *Biochem. Biophys. Res. Commun. 62,* 677.

Marcotte, P., and Walsh, C. (1976) *Biochemistry 15,* 3070.

Marcotte, P., and Walsh, C. (1978a), *Biochemistry 17,* 2864.

Marcotte, P., and Walsh, C. (1978b), *Biochemistry 17* (preceding paper in this issue).

Morasaki, M., and Bloch, K. (1972), *Biochemistry 11,* 309.

Nakano, M., Tsutsumi, Y., and Danowski, T. S. (1967), *Biochim. Biophys. Acta 139,* 40.

Orlowski, M., Reingold, D. F., and Stanley, M. E. (1977), *J. Neurochem. 28,* 349.

Rossi, A., and Schinz, H. (1948), *Helv. Chim. Acta 31,* 473.

Schneider, J. H., Cassir, R., and Chrodikian, F. (1960), *J. Biol. Chem. 235,* 1437.

Wiss, O., and Fuchs, H. (1952), *Helv. Chim. Acta 35,* 407.

Inactivation of Ribulosebisphosphate Carboxylase by Modification of Arginyl Residues with Phenylglyoxal†

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ABSTRACT: Phenylglyoxal rapidly and completely inactivates spinach and *Rhodospirillum rubrum* ribulosebisphosphate carboxylases. Inactivation exhibits pseudo-first-order kinetics and a reaction order of approximately one for both enzymes, suggesting that modification of a single residue per protomeric unit suffices for inactivation. Loss of enzymic activity is directly proportional to incorporation of [^{14}C]phenylglyoxal until only 30% of the initial activity remains. For both enzymes, extrapolation of incorporation to 100% inactivation yields 4–5 mol of [^{14}C]phenylglyoxal per mol protomer. Amino acid analyses confirm the expected 2:1 stoichiometry between phenylglyoxal incorporation and arginyl modification and suggest that other kinds of amino acid residues are not modified. (Thus, inactivation correlates with modification of 2–3 arginyl residues per protomer.) The substrate ribulose bisphosphate and some competitive inhibitors reduce the rates of inactivation of the carboxylases and prevent modification of about 0.5–1.0 arginyl residue per protomer. Inactivation is therefore a consequence of modification of a small number of residues out of the 35 and 29 total arginyl residues per protomer in spinach and *R. rubrum* carboxylases, respectively.

Because of its essentiality to the photosynthetic assimilation of CO_2, Rbl-P_2[1] carboxylase (EC 4.1.1.39) has been the subject of innumerable physiological, genetic, and biochemical studies (for a review, see Jensen & Bahr, 1977). A recent surge of interest in the mechanism and regulation of catalytic activity ensued with the realization that the carboxylase has an associated oxygenase activity which accounts for photorespiration (Bowes et al., 1971; Tolbert, 1973). Photorespiration is an energy-wasteful process which reduces the net amount of CO_2 fixation; when photorespiration is decreased by cultivating plants under low oxygen tension, plant yield can increase by

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[1] Abbreviations used: Rbl-P_2, D-ribulose 1,5-bisphosphate; PGO, phenylglyoxal; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; CR-P_2, 2-carboxyribitol 1,5-bisphosphate; Fru-P_2, D-fructose 1,6-bisphosphate; 3-PGA, D-3-phosphoglyceric acid; 6-PGlu, 6-phosphogluconic acid; butanediol-P_2, butane-1,4-diol 1,4-bisphosphate; EDTA, (ethylenedinitrilo)tetraacetic acid.

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50% or more (see reviews by Chollet & Ogren (1975) and by Zelitch (1975)). Thus, an understanding of the in vivo modulation of the carboxylase/oxygenase ratio and a determination of whether this ratio can be systematically manipulated by external means are of considerable agronomic significance.

Given the biological importance of Rbl-P_2 carboxylase and the ready availability of the enzyme [it comprises up to 50% of the soluble protein in green leaves (Kawashima & Wildman, 1970)], surprisingly little information about the active site of the enzyme is available. By the use of affinity labeling techniques, our laboratory has recently reported the isolation of several tryptic peptides from the spinach enzyme which are probably derived from the active-site region (Norton et al., 1975; Stringer & Hartman, 1978; Schloss et al., 1978b). Since two of these peptides contain arginyl residues and since arginyl residues are frequently involved in binding of anionic substrates (Riordan et al., 1977), we have investigated the reaction of the group-specific arginyl reagent phenylglyoxal with the carboxylase. The enzymes from two phylogenetically distant organisms, spinach and the photosynthetic bacterium *Rhodospirillum rubrum*, have been used in all experiments in an effort to determine if the results reflect species invariant structural features as might be expected for active-site modification. Because of gross differences in molecular architecture (McFadden & Tabita, 1974), the carboxylases from these two organisms provide an especially stringent system for the detection of common denominators in structure. Spinach Rbl-P_2 carboxylase has a molecular weight of 560 000 and is composed of eight 70 000-dalton protomeric units, each containing one large (56 000 daltons) and one small (14 000 daltons) subunit (Rutner, 1970; Siegel et al., 1972). In contrast, the *R. rubrum* enzyme is a simple dimer of identical 56 000-dalton subunits (Tabita & McFadden, 1974b) and is the smallest carboxylase known (McFadden & Tabita, 1974); each subunit is referred to as a protomer.

Experimental Procedures

Materials. Bicine, 3-PGA, 6-PGlu, phosphoglycerate phosphokinase, glyceraldehyde-3-phosphate dehydrogenase, glycerolphosphate dehydrogenase–triosephosphate isomerase, glutathione, and ATP were purchased from Sigma Chemical Co. Fru-P_2 was purchased from Boehringer Mannheim. PGO was obtained from Aldrich Chemical Co. and recrystallized twice from water prior to use. Butanediol-P_2, CR-P_2, and Rbl-P_2 were synthesized by published procedures (Hartman & Barker, 1965; Rabin et al., 1958; Horecker et al., 1958).

Spinach Rbl-P_2 carboxylase was isolated from freshly harvested spinach leaves by a slight modification (Norton et al., 1975) of the method of Wishnick & Lane (1971). *R. rubrum* (strain S-1) was cultured as described previously (Schloss & Hartman, 1977) and its Rbl-P_2 carboxylase isolated by a modification (Schloss et al., 1979) of the method of Tabita & McFadden (1974a). Both purified enzymes were homogeneous by disc gel electrophoresis.

[^{14}C]PGO was prepared from [^{14}C]acetophenone (ICN) by a published method (Riley & Gray, 1947), which was altered so as to omit the vacuum distillation. A mixture of dioxane (6 mL), water (0.2 mL), and selenium dioxide (0.4 g) was brought to boiling in a 100-mL three-necked flask equipped with thermometer, condenser, and stirrer. To the boiling mixture was added acetophenone (0.4 g, 500 μCi); after refluxing for 3 h, the reaction mixture was dried under a stream of nitrogen. Water (10 mL) was then added to the residue, and the resulting slurry was boiled for 5 min. After the addition of decolorizing charcoal, the hot (100 °C) slurry was filtered through Celite. Phenylglyoxal crystallized spontaneously; two recrystallizations gave 100 mg of analytically pure phenylglyoxal hydrate (330 000 dpm/μmol).

Assays. Carboxylase activity was determined at 25 °C by the spectrophotometric method of Racker (1963), as described in detail previously (Norton et al., 1975). Rbl-P_2 carboxylase concentrations were determined from A_{280nm}, assuming an $E^{1\%}_{1cm}$ of 16.4 (Wishnick & Lane, 1971) for the spinach enzyme and 9.74 (Tabita & McFadden, 1974b) for the *R. rubrum* enzyme. The specific activities of the spinach and *R. rubrum* carboxylases were 1.4 and 4.5 μmol of CO_2 per min per mg of protein, respectively. The latter value is higher than those published by other investigators which range from 0.48 to 3.3 units/mg (Anderson & Fuller, 1969; Ryan et al., 1974; Tabita & McFadden, 1974a; Whitman & Tabita, 1978). The increased specific activity that we observe in comparison to the highest (i.e., 3.3 units/mg) previously reported (Whitman & Tabita, 1978) is due primarily to the higher concentration of bicarbonate (66 mM instead of 20 mM) used in our assay.

Radioactivity. A Packard 3003 liquid scintillation spectrometer was used to measure radioactivity. Samples were mixed with the Triton X-100/toluene (1:2 v/v) cocktail of Patterson & Greene (1965); the cocktail contained 5.5 g/L of Permablend I (Packard) as scintillant.

Incorporation. Incorporation of [^{14}C]PGO into spinach or *R. rubrum* carboxylase was assayed as acid-insoluble radioactivity with the paper-disc method of Bollum (1968); application of this procedure to the carboxylase has been described in detail previously (Schloss et al., 1978b). Validity of the assay was verified by counting samples of dialyzed, [^{14}C]PGO-labeled enzyme both free in solution and after precipitation on filter paper discs.

Amino Acid Analyses. Complete acid hydrolysis of native and PGO-modified enzyme samples was achieved with 6 N HCl/0.1 M 2-mercaptoethanol at 110 °C for 21 h. PGO-modified enzyme was diluted (1:50) directly into hydrolysis mixtures (final PGO concentration $\lesssim$ 0.05 mM). Removal of unreacted PGO prior to hydrolysis was not necessary, since control samples hydrolyzed with and without PGO (0.05 mM) gave identical results. Hydrolysates were chromatographed on a Beckman 121M amino acid analyzer using the manufacturer's "3-h-single-column" system.

PGO Modification of Spinach and R. rubrum Carboxylase. Enzyme (0.5–5 mg/mL) was incubated with various concentrations of PGO (0.5–10 mM) at 21 or 30 °C in metal-free (by passage through Bio-Rad Chelex 100) 66 mM $NaHCO_3$/50 mM Bicine–NaOH/0.1 mM EDTA (pH 8.0) with or without $MgCl_2$ (5 mM) as indicated for each experiment. Periodically, aliquots were withdrawn and added directly to assay cuvettes or added to a quenching solution comprised of the Bicine buffer containing 0.2 M arginine prior to assay. The total ionic strength of modification reaction mixtures was adjusted to 0.128 with NaCl. All compounds that were tested as protectors against PGO inactivation were used as their sodium salts. In a few experiments $NaHCO_3$ was omitted, and the ionic strength was readjusted by the appropriate addition of NaCl.

Results

Inactivation of both spinach and *R. rubrum* carboxylase by PGO appears pseudo-first-order as seen in Figure 1. Upon prolonged incubation (data not shown), complete inactivation is obtained for both enzymes (<1% activity remaining) as would be expected if a residue essential to substrate binding or catalysis were modified. The second-order rate constant (k)

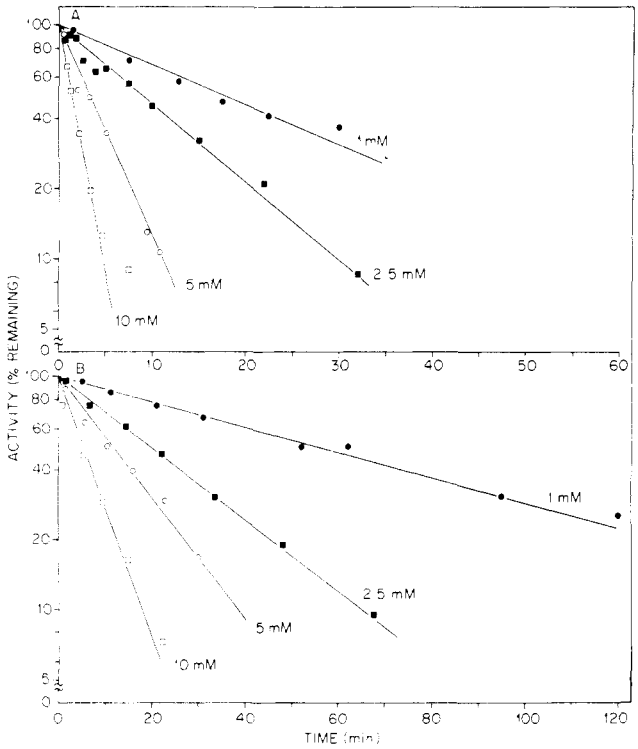

FIGURE 1: Pseudo-first-order inactivation of the spinach (A) and *R. rubrum* (B) carboxylases at 21 °C by 10 (□), 5 (○), 2.5 (■), or 1 (●) mM PGO in buffer containing 5 mM $MgCl_2$ (see Experimental Procedures). The activities of control samples remained constant for the time intervals shown.

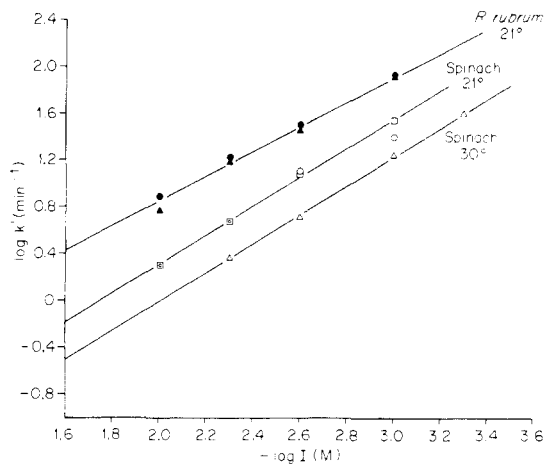

FIGURE 2: Plots of −log of the pseudo-first-order rate constant (k') against −log of PGO concentration (I). *R. rubrum* enzyme was modified at 21 °C in the presence (●) and absence (▲) of 5 mM $MgCl_2$. Spinach enzyme was modified at 30 °C in the absence of Mg^{2+} (△) and at 21 °C in the presence (○) and absence (□) of 5 mM $MgCl_2$. The first-order rate constants for inactivations carried out at 21 °C in the presence of Mg^{2+} were calculated from data depicted in Figure 1. The rate constants for inactivations carried out at 21 and 30 °C (in the absence of Mg^{2+}) were calculated from data not shown. Second-order rate constants are 5.1 M^{-1} s^{-1} (30 °C) and 2.5 M^{-1} s^{-1} (21 °C) for the spinach enzyme and 0.31 M^{-1} s^{-1} (21 °C) for the *R. rubrum* enzyme.

and reaction order (n) with respect to PGO concentration ([I]) can be obtained by replotting data shown in Figure 1 according to

$$\log k' = \log k + n \log [I] \qquad (1)$$

where k' is the apparent first-order rate constant at PGO concentration [I]. Such replots, shown in Figure 2, yield reaction orders of 1.24 and 1.06 for the spinach and *R. rubrum* enzymes, respectively. Taken together with the observation that the inactivation of both enzymes is pseudo-first-order, these reaction orders suggest that modification of a single amino acid residue per enzymic active site is sufficient for inactivation. Furthermore, the direct proportionality between log k' and log [I], even at very high concentrations of PGO, is consistent with inactivation occurring concomitantly with modification rather than subsequent to a rapid chemical event followed by a rate-limiting conformational change.

Mg^{2+} and CO_2 are allosteric activators of the carboxylase, and in their absence the enzyme exists in an inactive conformer (Chu & Bassham, 1975; Lorimer et al., 1976; Badger & Lorimer, 1976; Laing & Christeller, 1976). Furthermore, with fluorescent techniques Wildner (1976) has demonstrated a temperature-dependent conformational change for the carboxylase from spinach that takes place between 12 and 24 °C. In consideration of these effects, we examined the influences of Mg^{2+} and temperature on the reaction of carboxylase with PGO. Changes in temperature seem to have no unusual effect on the inactivation process, as a 9 °C increase (from 21 to 30 °C) approximately doubles the inactivation rate of the spinach enzyme without alteration of the reaction order (Figure 2). Lower temperatures were not examined because of time-dependent, reversible losses in enzymic activity of the spinach enzyme that occur in in the cold (Wildner, 1976; Ryan & Tolbert, 1975). Neither the rate of inactivation nor the reaction order of the spinach and bacterial enzymes is affected by Mg^{2+} (Figure 2). Although not examined in detail, the omission of $NaHCO_3$ from the reaction mixture reduces the rate of inactivation of the spinach enzyme by one-half (data not shown). Stimulation of the rate of inactivation by $NaHCO_3$ may not necessarily be a consequence of the allosteric properties of CO_2 but rather a direct stimulation of the rate of reaction of arginyl side chains with PGO (Cheung & Fonda, 1978).

The substrate Rbl-P_2 affords considerable protection against inactivation for both the spinach enzyme (Figure 3) and bacterial enzyme (not shown). If the spinach enzyme which has been modified with PGO in the presence or absence of Rbl-P_2 is subsequently dialyzed for 24 h at room temperature in the presence of CO_2/Mg^{2+}, the levels of activity obtained prior to dialysis are retained completely with little reversal (~10%) of PGO incorporation. This indicates that Rbl-P_2 protects against inactivation by preventing modification of a critical residue(s), rather than by conferring conformational stability on the modified enzyme. Despite the initial report of reversibility (Takahashi, 1968), irreversibility of PGO inactivation of several other enzymes has been observed (Riordan & Scandurra, 1975; Lobb et al., 1975; Lang et al., 1974).

The effects of Rbl-P_2 and several competitive inhibitors on the rate of inactivation of the spinach enzyme by PGO are shown in Table I. The protective effects of these compounds were assessed using the expression derived by Scrutton & Utter (1965)

$$k'_{prot}/k' = K_{prot}(1 - k'_{prot}/k')/P + k_2/k_1 \qquad (2)$$

where k'_{prot} and k' are the pseudo-first-order rate constants in the presence and absence of protector (P is protector concentration), respectively. K_{prot} is the dissociation constant for the enzyme·protector complex, and k_2/k_1 is the ratio of pseudo-first-order rate constants for inactivation of enzyme·protector complex and free enzyme. Rbl-P_2 appears to give complete protection against PGO inactivation. The inhibitors give either no protection, as shown for Fru-P_2 and butanediol-P_2, or only partial protection, as shown for 3-PGA and

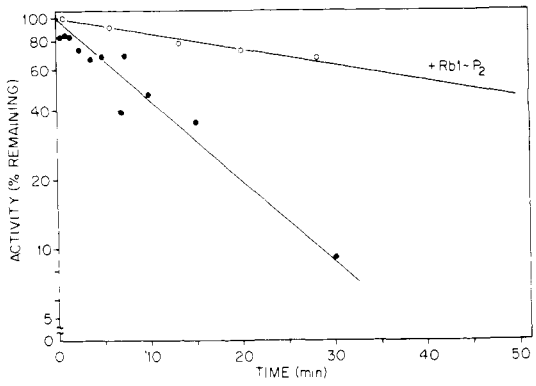

FIGURE 3: Modification of the spinach Rbl-P_2 carboxylase by 2.5 mM PGO in the presence (○) and absence (●) of 1 mM Rbl-P_2 at 21 °C (Mg^{2+}-free buffer).

TABLE I: Protection of Spinach Ribulosebisphosphate Carboxylase against Phenylgloxal Inactivation.

protector	concn tested (mM)	k_2/k_1	K_{prot} (mM)	K_m or K_i (mM)
ribulose bisphosphate	0.1, 1.0	−0.04	0.22	0.075[a] (0.006)[b]
fructose 1,6-bisphosphate	10	1	no protection	0.84[a]
3-phosphoglycerate	1.0, 10, 50	0.34	2.2	4.8[a]
6-phosphogluconate	1.0, 10	0.31	0.43	0.075[a]
butanediol 1,4-biphosphate	25	1	no protection	4.8[a]

[a] Determined in the present study. [b] Value in parentheses is the K_D for ribulose bisphosphate (Wishnick et al., 1970).

6-PGlu. The latter two compounds have a maximal effect of reducing the inactivation rate to one-third of that seen with the free enzyme. The lack of complete protection by all compounds tested except Rbl-P_2 could be interpreted to indicate that only Rbl-P_2 interacts strongly (presumably electrostatically) with the arginyl residue whose modification correlates with inactivation.

In addition to being competitive inhibitors, Fru-P_2 and 6-PGlu are also activators of the spinach enzyme, leading Chu & Bassham (1975) to propose an independent allosteric site for binding of these phosphate esters. The lack of protection (or of complete protection) by these compounds argues that inactivation is not a result of modification of such an allosteric site.

The dissociation constant obtained for Rbl-P_2 protection (0.22 mM) is somewhat higher than the K_m for this substrate (0.075 mM) and considerably higher than the dissociation constant (0.006 mM) reported by Wishnick et al. (1970). This discrepancy may be due to the fact that the K_m is obtained in the presence of CO_2 and Mg^{2+}, the dissociation constant in the absence of added CO_2 and Mg^{2+}, and the apparent dissociation constant from protection data in the presence of CO_2 (as added bicarbonate) alone.

The allosteric effects of CO_2 and Mg^{2+} have already been mentioned. Chu & Bassham (1975) have also proposed an allosteric site for Rbl-P_2 which functions to retard activation of the enzyme by CO_2 and Mg^{2+}. They regard the low dissociation constant obtained by Wishnick et al. (1970) to reflect binding to this allosteric site. If this is true, the rather large

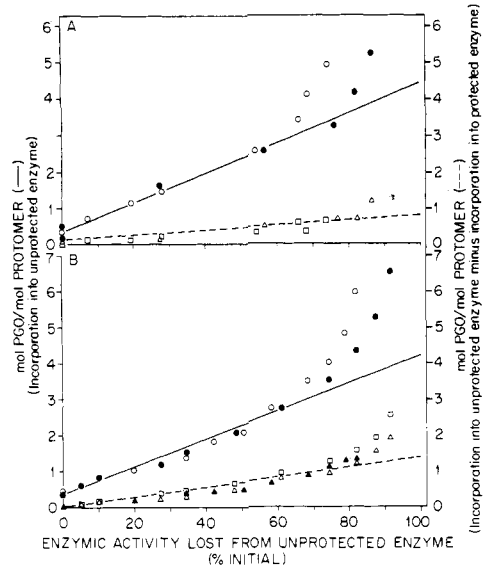

FIGURE 4: Incorporation of PGO per protomeric unit of spinach and *R. rubrum* carboxylases as a function of enzymic activity lost. (A) Spinach enzyme (5 mg/mL) inactivated by 0,5 mM [^{14}C]PGO in the presence (○) and absence (●) of 5 mM $MgCl_2$. The difference in incorporation between these samples and samples containing 1 mM Rbl-P_2 without Mg^{2+} (△) or 0.2 mM CR-P_2 with Mg^{2+} (□) at corresponding time points is shown in the lower dashed curve. (B) *R. rubrum* enzyme (5 mg/mL) inactivated by 2.5 mM [^{14}C]PGO in the presence (○) and absence (●) of 5 mM $MgCl_2$. The difference in incorporation between these samples and samples containing 1 mM Rbl-P_2 (△) or 0.5 mM CR-P_2 in the absence of Mg^{2+} (□) or 0.5 mM CR-P_2 in the presence of Mg^{2+} (▲) at corresponding time points is shown in the lower dashed curve.

K_{prot} observed in the present study might be used as an argument that protection is afforded by binding to the catalytic site rather than to an allosteric site.

Incorporation of [^{14}C]PGO into the spinach or *R. rubrum* carboxylase as a function of activity lost is shown in Figure 4. The results obtained with both enzymes are strikingly similar. The degree of inactivation is directly proportional to the extent of incorporation during the initial 60–70% loss of enzymic activity. Extrapolation of the linear portion of the curve to total inactivation yields between 4 and 5 PGO molecules per protomeric unit (70 000 daltons for the spinach enzyme and 56 000 daltons for the *R. rubrum* enzyme; see introductory section). Comparisons of amino acid compositions between native and PGO-inactivated enzyme reveal differences only in the levels of arginine; a sample of the spinach enzyme that is 75% inactivated in the presence of Mg^{2+} contains 5.0 mol of PGO/mol of protomer and shows a loss of 2.8 (±0.1 as standard error) arginyl residues/protomer. Similar results are obtained with both species of enzymes irrespective of the presence or absence of Mg^{2+}. Thus, as expected from the work of Takahashi (1968, 1977a,b) and other investigators (Daemen & Riordan, 1974; Lange et al., 1974; Lobb et al., 1975), 2 mol of phenylglyoxal are incorporated for each mol of arginine lost. Direct titration of free sulfhydryl groups in the proteins before hydrolysis demonstrates their lack of reaction with PGO.

In addition to the total incorporation that occurs during inactivation, also illustrated in Figure 4 is the protection against PGO modification afforded by Rbl-P_2 and CR-P_2. Samples containing Rbl-P_2 lost little activity during the period required for inactivation of the corresponding unprotected samples; enzymic activity in samples containing CR-P_2 was not monitored due to the formation of an inactive complex between this transition state analogue and enzyme (Siegel & Lane, 1972). At identical time points, aliquots were withdrawn

from both the unprotected and protected samples and assayed for protein-bound radioactivity. The differences in level of incorporation between unprotected and protected samples, determined at each time point, are plotted as a function of the percentage loss of enzymic activity from the unprotected enzyme. Extrapolation to complete inactivation demonstrates a difference of about one PGO per protomer (one-half an arginyl residue). Thus, of the total arginyl residues modified by PGO (2–3 per protomer), less than one is protected by Rbl-P_2 or CR-P_2; presumably inactivation is a consequence of the modification of the residues (less than one per protomer) subject to protection.

Discussion

Clearly, the inactivation of Rbl-P_2 carboxylase by PGO is a consequence of arginyl modification. Based on the previously determined stoichiometry of the reaction of guanidinium groups with PGO (Takahashi, 1968, 1977a), the total incorporation of [^{14}C]PGO into the carboxylase is accounted for by the decrease in arginine content. The lack of substantial reversibility of modification upon dialysis, as judged by alterations either in enzymic activity or ^{14}C incorporation, appears to exclude the possibility that inactivation results from a derivatization which escaped detection due to extreme lability.

Not only is PGO specific for arginyl residues of Rbl-P_2 carboxylase, but the reaction of the compound is restricted to a small percentage of the total arginyl residues present. The spinach and *R. rubrum* carboxylases contain 35 and 29 residues of arginine per protomer, respectively (Siegel et al., 1972; Tabita & McFadden, 1974b); of these, only 2–3 are readily accessible to PGO.

The sensitivity of the carboxylase toward PGO (with a protomer concentration of 70 μM and a PGO concentration of 500 μM, 80% inactivation occurs within several hours), the small number of residues whose modification leads to inactivation, the pseudo-first-order loss of activity at high molar ratios of reagent and enzyme, and the reaction order of about one with respect to PGO suggest the modification of an active-site arginyl residue. However, the protection data are not subject to unequivocal interpretation. Less than one residue per protomer is protected; nevertheless, protection against inactivation does appear to be a direct consequence of the prevention of the condensation of a residue(s) with PGO, since the protector can be removed from the derivatized enzyme without further loss of activity. Apparent correlation of inactivation with the modification of less than one residue equivalent was also observed in the reactions of 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate and pyridoxal phosphate with Rbl-P_2 carboxylase (Norton et al., 1975; Schloss & Hartman, 1977; Whitman & Tabita, 1978). Possible explanations are half-of-sites reactivity (Levitzki & Koshland, 1976) or more trivially that the carboxylase preparations are only partially active. Since similar results have now been obtained with several reagents and the carboxylases from two different species, the likelihood is increased that the former explanation is correct.

Another puzzling observation is the variable degree of protection afforded by saturating levels of different competitive inhibitors (Table I). In view of the complexities of the protection patterns, our studies neither refute nor verify the possibility of the involvement of an arginyl residue(s) in the binding of Rbl-P_2 by the carboxylase. However, the striking similarities, in all facets inspected, between the reactions of the phylogenetically and structurally dissimilar carboxylases from spinach and *R. rubrum* with PGO likely reflect a structural feature common to both species of enzyme. Whether this feature is the active site or a region essential to the maintenance of native tertiary structure remains to be seen.

During the latter stages of these studies, Lawlis & McFadden (1978) published preliminary results on the reactions of 2,3-butanedione with Rbl-P_2 carboxylases from barley and *Pseudomonas oxalaticus*. Although they interpreted the data as consistent with the modification of active-site arginyl residues, uncertainties were noted due to lack of reagent specificity. In the case of the bacterial enzyme, butanedione modified 22 of the 36 arginyl residues present per protomer; 3-PGA protected 8 residues per protomer. We believe that the much greater selectivity of PGO in comparison with butanedione establishes the carboxylase derivatized by PGO as the better choice for characterization at the level of proteolytic digests. Although the lability of the guanidinium–PGO adduct complicates the isolation of peptides, success was achieved in the isolation of a derivatized peptide from digests of alcohol dehydrogenase that had been inactivated with PGO (Jörnvall et al., 1977).

References

Anderson, L. E., & Fuller, R. C. (1969) *J. Biol. Chem. 244*, 3105–3109.

Badger, M. R., & Lorimer, G. H. (1976) *Arch. Biochem. Biophys. 175*, 723–729.

Bollum, F. J. (1968) *Methods Enzymol. 12B*, 169–173.

Bowes, G., Ogren, W. L., & Hageman, R. H. (1971) *Biochem. Biophys. Res. Commun. 45*, 716–722.

Cheung, S.-T., & Fonda, M. L. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1511.

Chollett, R., & Ogren, W. L. (1975) *Bot. Rev. 41*, 137–179.

Chu, D. K., & Bassham, J. A. (1975) *Plant Physiol. 55*, 720–726.

Daemen, F. J. M., & Riordan, J. F. (1974) *Biochemistry 13*, 2865–2871.

Hartman, F. C., & Barker, R. (1965) *Biochemistry 4*, 1068–1075.

Horecker, B. L., Hurwitz, J., & Weissbach, A. (1958) *Biochem. Prep. 6*, 83–90.

Jensen, R. G., & Bahr, J. T. (1977) *Annu. Rev. Plant Physiol. 28*, 379–400.

Jörnvall, H., Lange, L. G., III, Riordan, J. F., & Vallee, B. L. (1977) *Biochem. Biophys. Res. Commun. 77*, 73–78.

Kawashima, N., & Wildman, S. G. (1970) *Annu. Rev. Plant Physiol. 21*, 325–358.

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

Lange, L. G., III, Riordan, J. F., & Vallee, B. L. (1974) *Biochemistry 13*, 4361–4370.

Lawlis, V. B., & McFadden, B. A. (1978) *Biochem. Biophys. Res. Commun. 80*, 580–585.

Levitzki, A., & Koshland, D. E., Jr. (1976) *Curr. Top. Cell. Regul. 10*, 1–40.

Lobb, R. R., Stokes, A. M., Hill, H. A. O., & Riordan, J. F. (1975) *FEBS Lett. 54*, 70–72.

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

McFadden, B. A., & Tabita, F. R. (1974) *Biosystems 6*, 93–112.

Norton, I. L., Welch, M. H., & Hartman, F. C. (1975) *J. Biol. Chem. 250*, 8062–8068.

Patterson, M. S., & Greene, R. C. (1965) *Anal. Chem. 37*, 854–857.

Rabin, B. R., Shaw, D. F., Pon, N. G., Anderson, J. M., & Calvin, M. (1958) *J. Am. Chem. Soc. 80,* 2528–2532.
Racker, E. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) pp 188–190, Academic Press, New York.
Riley, H. A., & Gray, A. R. (1943) in *Organic Syntheses,* Collect. Vol. II (Blatt, A. H., Ed.) pp 509–511, Wiley, New York.
Riordan, J. F., & Scandurra, R. (1975) *Biochem. Biophys. Res. Commun. 66,* 417–424.
Riordan, J. F., McElvany, K. D., & Borders, C. L., Jr. (1977) *Science 195,* 884–886.
Rutner, A. C. (1970) *Biochem. Biophys. Res. Commun. 39,* 923–929.
Ryan, F. J., Jolly, S. O., & Tolbert, N. E. (9174) *Biochem. Biophys. Res. Commun. 59,* 1233–1241.
Ryan, F. J., Jolly, S. O., & Tolbert, N. E. (9174) *Biochem. Biophys. Res. Commun. 59,* 1233–2341.
Schloss, J. V., & Hartman, F. C. (1977) *Biochem. Biophys. Res. Commun. 75,* 320–328.
Schloss, J. V., Norton, I. L., Stringer, C. D., & Hartman, F. C. (1978a) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37,* 1310.
Schloss, J. V., Stringer, C. D., & Hartman, F. C. (1978b) *J. Biol. Chem. 253,* 5707–5711.
Schloss, J. V., Phares, E. F., Long, M. V., Norton, I. L., Stringer, C. D., & Hartman, F. C. (1979) *J. Bacteriol.* (in press).
Scrutton, M. C., & Utter, M. F. (1965) *J. Biol. Chem. 240,* 3714–3723.
Siegel, M. I. & Lane, M. D. (1972) *Biochem. Biophys. Res. Commun. 48,* 508–516.
Siegel, M. I., Wishnick, M., & Lane, M. D. (1972) *Enzymes, 3rd Ed., 6,* 169–192.
Stringer, C. D., & Hartman, F. C. (1978) *Biochem. Biophys. Res. Commun. 80,* 1043–1048.
Tabita, F. R., & McFadden, B. A. (1974a) *J. Biol. Chem. 249,* 3453–3458.
Tabita, F. R., & McFadden, B. A. (1974b) *J. Biol. Chem. 249,* 3459–3464.
Takahashi, K. (1968) *J. Biol. Chem. 243,* 6171–6179.
Takahashi, K. (1977a) *J. Biochem. (Tokyo) 81,* 395–402.
Takahashi, K. (1977a) *J. Biochem. (Tokyo) 81,* 403–414.
Tolbert, N. E. (1973) *Curr. Top. Cell. Regul. 7,* 21–50.
Whitman, W. B., & Tabita, F. R. (1978) *Biochemistry 17,* 1288–1293.
Wildner, G. F. (1976) *Z. Naturforsch. C 31,* 267–271.
Wishnick, M., & Lane, M. D. (1971) *Methods Enzymol. 23,* 570–577.
Wishnick, M., Lane, M. D., & Scrutton, M. C. (1970) *J. Biol. Chem. 245,* 4939–4947.
Zelitch, I. (1975) *Annu. Rev. Biochem. 44,* 123–145.