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INACTIVATION OF CRYSTALLINE TOBACCO RIBULOSEBISPHOSPHATE CARBOXYLASE BY MODIFICATION OF ARGININE RESIDUES WITH 2,3-BUTANEDIONE AND PHENYLGLYOXAL *

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Summary

Crystalline tobacco ribulosebisphosphate carboxylase (3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1 1.39) is rapidly and completely inactivated by 2,3-butanedione in borate buffer or phenylglyoxal, reagents which are highly specific for the modification of arginine residues. Inactivation by phenylglyoxal is enhanced in Bicine buffer and partially reversible, whereas inactivation by butanedione is markedly enhanced in borate buffer, irreversible in the presence of borate and partially reversed upon complete removal of borate and excess reagent.When the modification reaction is performed in the presence of various ligands, only the substrate ribulosebisphosphate and the diphosphorylated competitive inhibitor sedoheptulosebisphosphate protect against inactivation Loss of carboxylase activity is directly proportional to incorporation of [^{14}C]phenylglyoxal until about 15% of the initial activity remains. Extrapolation to zero activity suggests that inactivation by [^{14}C]phenylglyoxal correlates with the modification of three arginine residues per 69 000 dalton protomer. Complete protection by ribulosebisphosphate or sedoheptulosebisphosphate correlates with the shielding of 1—2 (1.27 ± 0.25) essential arginyl groups per protomer, which are located within the 55 000 dalton catalytic subunits of the protein. Similarly, amino acid analyses of acid hydrolysates of the butanedione- or

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Abbreviations ribulose-P_2, ribulose 1,5-bisphosphate, Nbs_2, 5,5′-dithiobis-(2-nitrobenzoate), sedoheptulose-P_2, sedoheptulose 1,7-bisphosphate, SDS, sodium dodecyl sulfate, Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, Bicine, *N,N*-bis(2-hydroxyethyl)glycine, Tricine, *N*-tris(hydroxymethyl)methylglycine

phenyl-glyoxal-inactivated and substrate-protected enzymes suggest that complete protection by ribulosebisphosphate correlates with the shielding of 1 9—2 4 arginine residues per protomer However, modification of the control and substrate-protected enzymes with these arginine-selective α-dicarbonyls does not alter modulation by anionic effectors

Introduction

Ribulosebisphosphate carboxylase (3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1.1 39) catalyzes the initial reaction in the C_3 photosynthetic carbon reduction cycle, the carboxylation of ribulose-P_2 to yield two molecules of 3-*P*-glycerate The same protein, functioning as an internal monooxygenase, also catalyzes the initial reaction in the C_2 photorespiratory carbon oxidation cycle, the oxygenation of ribulose-P_2 to yield 3-*P*-glycerate and 2-*P*-glycolate, the immediate precursor of the photorespiratory substrate glycolic acid [1,2]. The enzyme has been obtained in a highly purified crystalline state from tobacco [3] and spinach [4] leaves and is a large protein ($M_r \approx 550\,000$) composed of eight 69 000 dalton protomers, each consisting of one large (55 000 dalton) catalytic subunit and one small (14 000 dalton) non-catalytic subunit [5]

Although it is generally recognized that ribulosebisphosphate carboxylase-oxygenase plays a pivotal role in controlling the balance between photosynthetic CO_2 fixation and photorespiration in leaf tissue of most major agronomic crops [1,2], surprisingly little is known about the amino acid residues involved in either the catalytic functions of this important protein or the regulation of these functions by effector molecules. With the recent development and use of α-dicarbonyl compounds as selective reagents for the chemical modification of arginyl groups under mild conditions, an ever-increasing awareness of the importance of arginine residues in binding phosphorylated substrates, coenzymes and effectors in a wide variety of enzymes has emerged [6,7] Indeed, McFadden's [8] and Hartman's [9] groups have published results on the modification of bacterial and higher plant ribulosebisphosphate carboxylases by arginine-selective α-dicarbonyls. Lawlis and McFadden [8] reported that the higher plant-type enzyme isolated from *Pseudomonas oxalaticus* and barley is inactivated by 2,3-butanedione. The borate-dependent inactivation was markedly reduced by high levels (10—20 mM) of the product 3-*P*-glycerate, suggestive of the modification of active-site arginyl groups, but the effect of the substrate ribulose-P_2 was not examined. In addition, uncertainties were noted due to the lack of reagent specificity for essential arginine residues. In the case of the *P oxalaticus* enzyme, an 80% loss of activity was associated with the modification of 22 of the 36 arginine residues present per protomer and complete protection by 3-*P*-glycerate correlated with the shielding of 15 residues per protomer. In a related study, Schloss et al. [9] reported that phenylglyoxal completely and irreversibly inactivates spinach and *Rhodospirillum rubrum* ribulosebisphosphate carboxylases. Inactivation of the spinach carboxylase was markedly reduced by the substrate ribulose-P_2, whereas a variety of phosphorylated competitive inhibitors (including 6-*P*-gluconate, 3-*P*-gly-

cerate, fructose-1,6-P_2 and butanediol-1,4-P_2) afforded little or no protection. Of the 2—3 total arginine residues modified per protomer by phenylglyoxal, only about 0.5 arginine/protomer was shielded by ribulose-P_2 or the transition-state analogue 2-carboxypentitol 1,5-P_2, suggestive of half-of-the-sites reactivity [9]. In view of the complexities of these protection data, it was concluded that these experiments neither refute nor verify the possibility of the involvement of an arginine residue(s) in the binding of ribulose-P_2 by the carboxylase [9].

Since ribulosebisphosphate carboxylase not only acts on a phosphorylated substrate, but is also modulated by anionic effectors including NADPH and 6-*P*-gluconate [10,11], we have further investigated the possible importance of arginine residues in the catalytic and regulatory properties of the crystalline tobacco enzyme by modification with the arginine-selective α-dicarbonyls 2,3-butanedione [12] and phenylglyoxal [13—15].

Preliminary reports of some of the findings have appeared [16,17].

Materials and Methods

Materials. 2,3-Butanedione, 99%, was purchased from Aldrich Chemical Co. and used without further purification. Phenylglyoxal hydrate was obtained from Tridom Chemical Inc. and [2-^{14}C]phenylglyoxal (30 Ci/mol) was purchased from Research Products International Corp. The stated radiochemical purity of the [^{14}C]phenylglyoxal (99%) was verified by one-dimensional chromatography on precoated silica gel plates using ethyl acetate/petroleum ether, 35—60°C (1 : 1) as the solvent [18]. Buffers, Nbs_2 and the sodium salts of ribulose-P_2, NADPH, 6-*P*-gluconate and sedoheptulose-P_2 were obtained from Sigma Chemical Co. $NaH^{14}CO_3$ was purchased from New England Nuclear and SDS from BioRad Labs. Sephadex G-50 (coarse) and G-100 and prepacked columns of Sephadex G-25 (medium; 9.1 ml bed vol.) were obtained from Pharmacia Fine Chemicals.

Enzyme preparation and assay Crystalline ribulosebisphosphate carboxylase was obtained from tobacco (*Nicotiana tabacum* L. cv. Xanthi) leaf homogenates and recrystallized twice as described previously [3]. The protein crystals were dissolved in 25 mM Hepes-NaOH/0.1 M NaCl (pH 8.0) to yield a final concentration of 13—20 mg/ml and the resultant enzyme solution was heat-activated for 20 min at 50°C [19]. Carboxylase activity was determined by $^{14}CO_2$ incorporation at 30°C in the presence of $NaH^{14}CO_3$ and ribulose-P_2, essentially as described by Kung et al [3]. For routine assays the complete reaction mixture contained 0.1 M Tris/10 mM $MgCl_2$/0.1 mM Na_2EDTA/0.5 mg/ml Fraction V bovine albumin (entire solution adjusted to pH 8.0 with HCl)/0.5 mM freshly dissolved ribulose-P_2/25 mM $NaH^{14}CO_3$ (0.25 Ci/mol)/5—10 μg of protein in a final volume of 0.5 ml. Following a 10 min preincubation of the protein at 30°C in the presence of Mg^{2+} and $H^{14}CO_3^-$ (to CO_2/Mg^{2+}-activate the enzyme), the reactions were initiated with ribulose-P_2 and terminated within 30—60 s by injecting 0.1 ml 6 M acetic acid. Blanks were treated in an identical manner except that no ribulose-P_2 was added. To assess modulation of the control, modified and substrate-protected enzyme activity by anionic effectors, aliquots of the gel-filtered protein samples (see below) were immediately assayed for carboxylase activity following 10 min preincubation in the Tris-buffered reac-

tion mixture (pH 7.6 with HCl)/10 mM Mg^{2+}/1 mM $NaH^{14}CO_3$ (2 5 Ci/mol)/± effector [11].

Ribulosebisphosphate carboxylase was purified to homogeneity from glutamate/CO_2/thiosulfate-grown *Thiobacillus intermedius* exactly as described by Bowman and Chollet [20]. Prior to chemical modification by phenylglyoxal (see below), aliquots of this higher plant-type carboxylase [20] were dialyzed overnight at 4°C against 4000 vol. 25 mM Hepes/1 mM Na_2EDTA/2 mM dithiothreitol/0.6 M sucrose (pH 8.0 with NaOH) to remove interfering Tris, Mg^{2+} and HCO_3^- present in the original sucrose gradient fractions.

Protein concentration, as mg/ml, was estimated spectrophotometrically by multiplying A_{280nm}^{1cm} by the factor 0.70 for the tobacco enzyme [3] and 0.84 for *T intermedius* [20]. The molar concentration of the tobacco carboxylase was calculated using a molecular weight of 550 000 [19].

Inactivation by 2,3-butanedione. Inactivation was routinely performed by adding an aliquot of freshly prepared 40 mM butanedione in 0 1 M $Na_4B_4O_7$ buffer (readjusted to pH 8 0 with NaOH) to an equal volume of protein solution which had been preincubated for 15 min at 30°C in the absence or presence of various ligands (prepared in 25 mM Hepes buffer readjusted to pH 8.0) The final solution contained 50 mM borate (pH 8.0)/25 mM NaCl/12 mM Hepes/6—8 μM enzyme/20 mM butanedione and various ligands as specified. Controls were treated identically except that no butanedione was added. After the specified times at 30°C, 0.2-ml aliquots were passed through a small column of Sephadex G-25 equilibrated with 10 mM borate/0.1 M NaCl (pH 7.5) Aliquots of the protein eluant were either diluted with 4 vol. 50 mM Tris-HCl/10 mM NaCl (pH 7.5) and immediately assayed for carboxylase activity or added to an equal volume of ice-cold 12 1 N HCl for subsequent hydrolysis and amino acid analysis (see below). Alternatively, at the appropriate times the inactivation reaction was terminated by diluting aliquots with 30 vol. 50 mM Tris-HCl/25 mM NaCl (pH 7.5). Preliminary experiments indicated that 30-fold dilution with Tris-NaCl buffer was effective in obtaining control or inactivated enzyme activity that was stable for at least 90 min at 30°C [16].

Inactivation by phenylglyoxal Unless noted otherwise, inactivation was performed by adding an aliquot of freshly prepared 10 mM phenylglyoxal in 0.1 M Bicine-NaOH buffer (pH 8.0) to an equal volume of protein solution which had been preincubated as described above. The final solution contained 50 mM Bicine (pH 8.0)/25 mM NaCl/12 mM Hepes/6—9 μM enzyme/5 mM phenylglyoxal and various ligands as specified in the figure and table legends. Controls were treated identically except that no phenylglyoxal was added. After the specified times at 30°C, 0.2-ml aliquots were gel filtered through Sephadex G-25 equilibrated with either 50 mM Bicine-NaOH/25 mM NaCl (pH 7.7) or 10 mM borate/0.1 M NaCl (pH 7.5) (for amino acid analyses). Aliquots of the protein eluant were either diluted with 4 vol. 50 mM Bicine-NaOH/25 mM NaCl (pH 7.7) and immediately assayed for carboxylase activity or added to an equal volume of ice-cold 12.1 N HCl for subsequent hydrolysis and amino acid analysis (see below). Alternatively, at the appropriate times the inactivation reaction was terminated by diluting aliquots with 30 vol. 50 mM Tris-HCl/10 mM NaCl (pH 7.5) or 50 mM Bicine-NaOH/10 mM NaCl (pH 7.7) and 50-μl samples were assayed immediately for activity. Phenylglyoxal concentration (mM) was

determined spectrophotometrically by diluting aliquots of the Bicine-buffered stock solution with 300 vol. water and dividing A^{1cm}_{253nm} by 12.6 [21].

Incorporation of [2-^{14}C]phenylglyoxal Modification of the tobacco enzyme by [^{14}C]phenylglyoxal was performed exactly as described above for inactivation except that unlabeled reagent was replaced with 10 mM [^{14}C]phenylglyoxal (0 5—1 25 Ci/mol) After the specified times at 30°C, 0.2-ml aliquots were passed through Sephadex G-25 equilibrated with 50 mM Bicine-NaOH/25 mM NaCl (pH 7.7). Aliquots of the ^{14}C-protein eluant were diluted with 4 vol column buffer and immediately assayed for [^{14}C]phenylglyoxal incorporation by liquid scintillation spectroscopy and for enzyme activity. The carryover of ^{14}C-protein into the ^{14}C-based carboxylase assays was corrected by taking into consideration the radioactivity in the minus-ribulose-P_2 blanks. The specific radioactivity of the [^{14}C]phenylglyoxal was determined experimentally by assaying aliquots of the stock solution for radioactivity and absorbance at 253 nm (see above). From the [^{14}C]phenylglyoxal-incorporation and -specific radioactivity determinations, the extent of arginyl modification per enzyme protomer was calculated assuming a 2 · 1 stoichiometry between reagent incorporation and arginine modification [9,13,14].

To assess [^{14}C]phenylglyoxal modification of the carboxylase at the subunit level, aliquots of the Sephadex G-25 eluant (see above) were added to an equal volume of ice-cold 10% (w/v) trichloroacetic acid and the precipitate was collected by centrifugation (2500 × *g*) at 4°C. The pellet was washed once with 2 ml of −20°C acetone and dried at 37°C. The ^{14}C-labeled protein precipitate (approx. 0.9 mg) was dissolved in 1.0 ml 50 mM Tris-HCl buffer (pH 9.0)/1% (w/v) SDS/0.14 M 2-mercaptoethanol and dissociated as described previously [20,22]. The dissociated subunits were separated at 25°C by descending chromatography through Sephadex G-100 equilibrated and eluted with 50 mM Tris-HCl buffer (pH 8.6)/0.5% SDS [20]. Aliquots of each 1.2-ml fraction were analyzed for radioactivity and absorbance at 280 nm.

Amino acid analyses For the determination of arginine modification by butanedione or phenylglyoxal, aliquots of the gel-filtered control, modified and substrate-protected enzymes (see above) were hydrolyzed in evacuated glass tubes at 110°C for 24 h with 6 N HCl and subjected to amino acid analysis on the short basic column of a Beckman 120C amino acid analyzer equipped with a computing integrator. Loss of arginine was determined by duplicate analysis of single samples using a value of 32.8 lysine/promoter as the basis of reference [23]. Unmodified tobacco carboxylase gave a value of 36.9 arginine/protomer for the average of 12 determinations, which compares favorably with the value of 36.2 reported by Kung et al. [23] for the crystalline tobacco protein.

Results and Discusssion

Modification of ribulosebisphosphate carboxylase by 2,3-butanedione Treatment of the crystalline tobacco enzyme with butanedione in borate buffer results in a time- and concentration-dependent loss of activity, although the inactivation reaction deviates from pseudo-first-order kinetics [16]. Analysis of the enzyme which has been inactivated 60% by incubation with 20 mM butanedione for 35 min reveals that the K_m value for ribulose-P_2 is slightly increased (from 99 μM in the control to 168 μM in the partially inactivated

sample), whereas the apparent maximal velocity at infinite substrate concentration (V) is decreased by 57%. Inactivation is markedly enhanced by borate buffer (relative to Hepes or Tris) and alkaline pH [16], in agreement with the findings of other butanedione inactivation studies [6,7]. In similar agreement (cf Ref 7) are the observations that the inactivation of ribulosebisphosphate carboxylase by butanedione is irreversible in the presence of borate and partially reversed upon complete removal of excess reagent and borate by gel filtration [16]. Although both the control and modified proteins are progressively inactivated by prolonged incubation in borate buffer at pH 7.5, the relative activity of the modified enzyme (expressed as a percentage of the corresponding control) remains constant for at least 3 h.

Inactivation of the carboxylase by butanedione is markedly reduced by preincubating the enzyme with the substrate ribulose-P_2 [16]. For example, upon prolonged incubation (3 h) in the presence of 20 mM butanedione complete inactivation is observed (<2.5% activity remaining), as would be expected if residue(s) essential to substrate binding or catalysis were modified. However, in the presence of 1 mM ribulose-P_2 only 35% of the activity is lost over the same period. In contrast, none of the non-substrate phosphorylated ligands examined, including the effectors NADPH and 6-*P*-gluconate and the reaction products 3-*P*-glycerate and 2-*P*-glycolate, significantly decrease inactivation [16]. Similarly, CO_2/Mg^{2+}-activation of the enzyme (in the absence or presence of NADPH or 6-*P*-gluconate) prior to modification has little influence on butanedione inactivation [16].

Butanedione is highly specific for the modification of arginine residues in proteins. The modification is enhanced by borate buffer, which likely stabilizes the *cis*-diol guanidinium-butanedione adduct, and is at least partially reversed upon removal of excess reagent and borate [7,12]. When the observations that inactivation of ribulosebisphosphate carboxylase by this α-dicarbonyl is enhanced by borate and partially reversed upon complete removal of borate are coupled to the results from the protection experiments, they strongly suggest that the modification of essential arginine residues within the binding domain for ribulose-P_2 (presumably at the active site) is responsible for inactivation.

TABLE I

CORRELATION OF BUTANEDIONE INACTIVATION OF TOBACCO RIBULOSEBISPHOSPHATE CARBOXYLASE WITH LOSS OF ARGININE

Modification of 8 1 μM enzyme by 20 mM butanedione (BD) was carried out for 65 min at 30° C in 50 mM borate buffer (pH 8 0) in the absence or presence of 1 mM ribulose-P_2 (Rbu-P_2) The results represent the mean of four independent experiments Control activity was 579 nmol $H^{14}CO_3^-$ fixed/min per mg protein

Enzyme	Carboxylase activity (% of control)	Arginine per protomer	Arginine modified per protomer	Arginine shielded/protomer for 100% protection *
Control	100	36 6		
BD-modified	20	27 2	9 4	
				2 4
Rbu-P_2-protected	74	28 5	8 1	

* Calculated from (difference in number of arginine groups modified)/(difference in % activity) × 100

Amino acid analyses of the control and partially inactivated enzymes were performed to identify the basic amino acid residues modified by treatment with butanedione. Whereas the ratio of histidine to lysine in samples incubated for 65 min in 20 mM butanedione is identical to that in the unmodified control, the ratio of arginine to lysine decreases 25% over the same period. More noteworthy, an 80% loss of activity is associated with the modification of 9—10 of the 37 arginine residues present per protomer (Table I). The excessive loss (about 25%) of arginine residues during the course of enzyme inactivation is indicative of a concomitant modification of non-essential arginyl groups. Since ribulosebisphosphate carboxylase contains about 290 arginine/mol [23], modification of arginine residues in addition to those essential for substrate binding or catalysis is not unexpected. Insight into the number of essential arginyl groups per protomer was obtained by comparing the number of residues modified and the percent carboxylase activity of samples treated with butanedione for the same length of time in the absence or presence of ribulose-P_2 (Table I). Assuming the difference in loss of arginyl groups is linearly related to the difference in activity between the inactivated and substrate-protected samples, the extrapolations suggest that complete protection by ribulose-P_2 correlates with the shielding of 2—3 arginine residues per protomer. However, given the limited sensitivity of amino acid analysis and the observation that a relatively large number of arginine groups are modified in either the presence or absence of substrate (Table I), this extrapolated value cannot be taken as unambiguous quantitative evidence for the number of essential arginine residues per protomeric unit of ribulosebisphosphate carboxylase.

Modification of ribulosebisphosphate carboxylase by phenylglyoxal Although the modification of essential arginine residues within the binding domain for ribulose-P_2 appears responsible for the complete inactivation of the tobacco carboxylase by 2,3-butanedione, several characteristics of the modification reaction between butanedione and this particular protein are undesirable from an experimental standpoint. These include the apparent lack of reagent specificity for essential arginine residues (Table I and Ref. 8), the anomalous inactivation kinetics [16] and the effect of modification on both V and K_m values (ribulose-P_2). Previous studies from this laboratory have indicated that hydrophobic reagents are especially useful probes for monitoring activity-associated events in the carboxylase protein, presumably due to the hydrophobic nature of the large catalytic subunits [19]. In view of these observations, we selected phenylglyoxal to further investigate the importance of arginine residues in the catalytic properties of ribulosebisphosphate carboxylase. Not only is this α-dicarbonyl quite specific for arginyl groups [13—15], but the modification reaction appears particularly sensitive to the chemical microenvironment (presumably the degree of hydrophobicity) of the arginine residues in proteins [24—27]. Thus, the preferential modification of a small number of specific arginyl groups in the hydrophobic catalytic subunits might be possible using phenylglyoxal in spite of the large number of arginine residues in the holoenzyme.

Bicarbonate buffer is widely used in phenylglyoxal modification studies [24,25,27,28] as it likely catalyzes a nucleophilic attack by the arginyl-guani-

dinium group against the carbonyl carbon of the reagent [7,29]. However, for our routine studies it was desirable to avoid the use of this buffer since CO_2 is both an activator and substrate for ribulosebisphosphate carboxylase [5] Comparison of several other systems with buffering capacity around pH 8 (Bicine, borate, Tricine, Tris) indicated that inactivation of the tobacco enzyme was markedly enhanced in Bicine buffer, whereas no inactivation was observed in Tris or Tricine. The total ineffectiveness of Tris buffer is expected since this amine is known to react with the reagent [13,29]. Inactivation of tobacco ribulosebisphosphate carboxylase by 2 6 or 5 1 mM phenylglyoxal in Bicine buffer follows pseudo-first-order kinetics until at least 85% of the activity is lost. Upon prolonged incubation (2.5 h) in the presence of 5 mM reagent complete inactivation is observed (<1% activity remaining) (cf., Table V). Analysis of the enzyme which had been inactivated about 60% by incubation with 5.3 mM phenylglyoxal for 25 min revealed that the K_m value for ribulose-P_2 is unchanged (102 μM in the control vs. 117 μM in the modified sample), whereas the apparent V value decreased 63% following modification. Thus, the activity of the partially inactive enzyme can be attributed to the unmodified protein still present rather than to the formation of a modified carboxylase with dif-

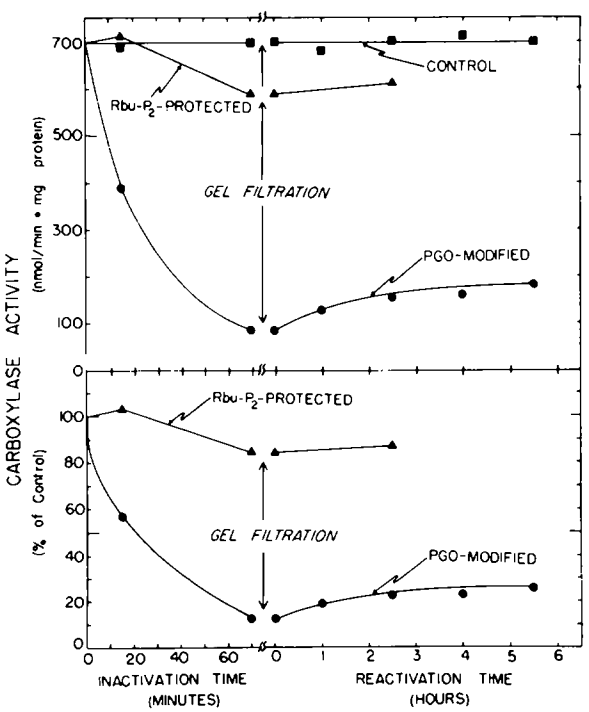


Fig 1 Reversibility of phenylglyoxal inactivation of tobacco ribulosebisphosphate carboxylase upon removal of excess reagent Modification of 8 2 μM enzyme by 5 mM phenylglyoxal (PGO) was carried out at 30° C in 50 mM Bicine buffer (pH 8 0) in the absence (●———●) or presence (▲———▲) of 1 mM ribulose-P_2 (Rbu-P_2) After a 15 or 70 min inactivation period an aliquot was passed through a Sephadex G-25 column equilibrated with 50 mM Bicine/25 mM NaCl (pH 7 7) and the protein eluant assayed for carboxylase activity immediately after and up to 5 5 h after gel filtration. The control (■———■) was subjected to identical conditions except that neither PGO nor Rbu-P_2 was added

TABLE II

EFFECT OF VARIOUS LIGANDS ON PHENYLGLYOXAL INACTIVATION OF *T INTERMEDIUS* AND TOBACCO RIBULOSEBISPHOSPHATE CARBOXYLASES

Modification of the *T intermedius* enzyme (85 μg/ml) by 5 2 mM phenylglyoxal was carried out at 30°C in 50 mM Bicine buffer (pH 8 0) in the absence or presence of 2 mM ribulose-P_2 (Rbu-P_2), sedoheptulose-P_2 (Sed 1,7-P_2) or 6-*P*-gluconate After a 70 min inactivation period an aliquot was passed through a Sephadex G-25 column equilibrated with 50 mM Bicine/1 mM Na_2EDTA/1 mM dithiothreitol (pH 8 0) and the protein eluant was immediately assayed for carboxylase activity Control activity was 1 70 μmol $H^{14}CO_3^-$ fixed/min per mg protein Modification of the tobacco enzyme (4 4 mg/ml) by 5 1 mM phenylglyoxal was carried out for 65 min at 30°C as described above Control activity was 590 nmol $H^{14}CO_3^-$ fixed/min per mg protein

Ligand preincubated	Carboxylase activity (% of control)	
	T intermedius	Tobacco
None	8	9
Rbu-P_2	69	79
Sed 1,7-P_2	64	69
6-*P*-gluconate	8	22

ferent properties. Phenylglyoxal inactivation of the tobacco enzyme is slowly and partially reversed upon complete removal of excess reagent by gel filtration (Fig. 1). The specific activity of the modified enzyme roughly doubles over a 5.5 h reactivation period at 30°C, whereas that of the control sample remains unchanged. A slow partial reactivation is also observed after the inactivation reaction is terminated by 30-fold dilution in Bicine- or Tris-NaCl buffer, with a 40—50% increase in carboxylase specific activity occurring after a 1.5 h incubation at 30°C. These results are in contrast to the observation of Schloss et al. [9] that the spinach and *R rubrum* carboxylases are inactivated irreversibly by this α-dicarbonyl. Partial reversibility of phenylglyoxal inactivation of several other enzymes has been reported previously [13,26,27,30].

As in the butanedione studies, the substrate ribulose-P_2 affords considerable protection against phenylglyoxal inactivation of the higher plant-type carboxylase isolated from *T intermedius* and tobacco (Table II, Fig. 1). The observation that the activity of the substrate-protected tobacco enzyme remains essentially constant for at least 2.5 h at 30°C following removal of ribulose-P_2 and excess reagent by gel filtration (Fig. 1) indicates that the substrate protects against inactivation by preventing modification of an essential residue(s), rather than by conferring conformational stability on the modified enzyme. Inactivation of both enzymes is also markedly reduced by preincubating the proteins with the diphosphorylated competitive inhibitor sedoheptulose-P_2 [31] (Table II). In contrast, 6-*P*-gluconate, an equally potent but monophosphorylated competitive inhibitor [9], provides little or no protection against inactivation (Table II and Ref. 9). Similarly, CO_2/Mg^{2+}-activation of the tobacco enzyme with 20 mM HCO_3^-/10 mM Mg^{2+} prior to modification has little influence on inactivation.

Given the specificity of phenylglyoxal for arginyl modification and the results from the protection experiments, the above observations suggest that the modification of essential arginine residues within the binding domain for ribulose-P_2 is responsible for the observed inactivation. Direct titration of free

TABLE III

CORRELATION OF PHENYLGLYOXAL INACTIVATION OF TOBACCO RIBULOSEBISPHOSPHATE CARBOXYLASE WITH LOSS OF ARGININE

Modification of 8 6 μM enzyme by 4 5 mM phenylglyoxal (PGO) was carried out for 65 min at 30°C in 50 mM Bicine buffer (pH 8 0) in the absence or presence of 1 mM ribulose-P_2 (Rbu-P_2) The results represent the mean of two independent experiments Control activity was 625 nmol $H^{14}CO_3^-$ fixed/min per mg protein

Enzyme	Carboxylase activity (% of control)	Arginine per protomer	Arginine modified per protomer	Arginine shielded/protomer for 100% protection *
Control	100	37 6		
PGO-modified	15	32 8	4 8	1 9
Rbu-P_2-protected	87	34 2	3 4	

* Calculated from (difference in number of arginine groups modified)/(difference in % activity) X 100

sulfhydryl groups with Nbs_2 in the presence of SDS [19] demonstrated that there is no irreversible loss of -SH residues in the inactivated or substrate-protected tobacco enzymes. Amino acid analyses of the control and modified proteins indicated that an 85% loss of carboxylase activity is associated with the

TABLE IV

CORRELATION OF INACTIVATION OF TOBACCO RIBULOSEBISPHOSPHATE CARBOXYLASE WITH INCORPORATION OF [2-^{14}C]PHENYLGLYOXAL

In Expt I, modification of 7 8 μM enzyme by 5 3 mM [2-^{14}C]phenylglyoxal (PGO) (0 79 Ci/mol) was carried out at 30°C in 50 mM Bicine buffer (pH 8 0) in the absence or presence of 2 mM ribulose-P_2 (Rbu-P_2) or 6-P-gluconate Control carboxylase activity was 659 nmol $H^{14}CO_3^-$ fixed/min per mg protein In Expt II, modification of 6 4 μM enzyme by 5 2 mM [2-^{14}C]phenylglyoxal (0 48 Ci/mol) was carried out as described above Control carboxylase activity was 462 nmol $H^{14}CO_3^-$ fixed/min per mg protein In Expt III, modification of 8 4 μM enzyme by 5 3 mM [2-^{14}C]phenylglyoxal (1 25 Ci/mol) was carried out as described above in the absence or presence of 2 mM Rbu-P_2 or sedoheptulose-P_2 (Sed 1,7-P_2) Control carboxylase activity was 940 nmol $H^{14}CO_3^-$ fixed/min per mg protein

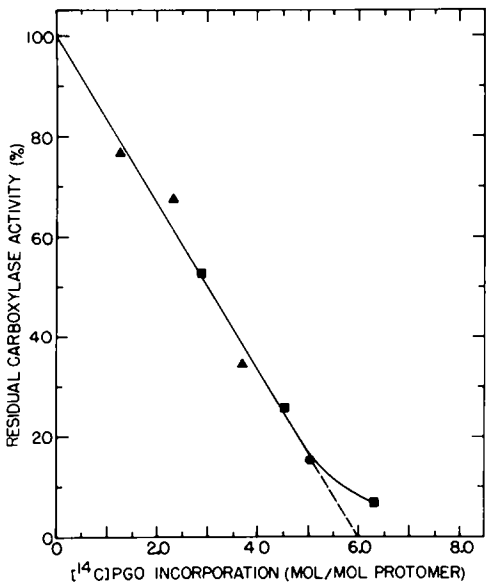
Expt No	Enzyme	Time at 30°C (min)	Carboxylase activity (% of control)	[^{14}C]PGO incorporation (mol/mol protomer)	Arginine shielded/ protomer for 100% protection *
I	[^{14}C]PGO-modified	5	77	1 27	
		12	67	2 32	
		28	34	3 69	
	Rbu-P_2-protected	5	102	0 73	1 1
		12	104	1 43	1 2
		28	97	2 45	1 0
	6-P-gluconate-protected	28	58	3 87	
II	[^{14}C]PGO-modified	15	52	2 87	
		35	25	4 53	
		65	6	6 31	
	Rbu-P_2-protected	15	97	1 83	1 2
		35	93	3 04	1 1
		65	76	4 14	1 6
III	[^{14}C]PGO-modified	45	15	5 03	
	Rbu-P_2-protected	45	79	3 31	1 3
	Sed 1,7-P_2-protected	45	78	2 87	1 7

* Calculated from (difference in [^{14}C]incorporated/protomer)/(difference in % activity) X 100, assuming a 2 1 stoichiometry between phenylglyoxal incorporation and arginine modification [9,13,14]

modification of about 5 of the 37 arginine residues present per protomer (Table III). These results indicate that of the two α-dicarbonyls used in the present study, phenylglyoxal is by far the more selective modifier of the carboxylase protein in that about twice as many total arginine residues are modified by butanedione compared to phenylglyoxal to achieve the same degree of inactivation (cf., Tables I and III and Refs. 8 and 9). However, as in the butanedione studies (Table I), comparison of the phenylglyoxal-modified and substrate-protected samples suggests that complete protection by ribulose-P_2 correlates with the shielding of about two arginine residues per protomer (Table III).

Besides the greater selectivity of phenylglyoxal as a chemical modifier of arginine groups in ribulosebisphosphate carboxylase, another advantage of this α-dicarbonyl is that the use of [^{14}C]phenylglyoxal permits a more accurate estimate of the stoichiometry of inactivation by reagent incorporation than can be obtained by analyzing for losses in arginine from a large initial number [7]. [^{14}C]Phenylglyoxal is rapidly incorporated into the tobacco enzyme with a concomitant decrease in carboxylase activity (Table IV). When the data in Table IV for the modified samples are plotted as percent activity vs. ^{14}C-incorporation per protomer (mol/mol), the resultant linear plot shows curvature after the enzyme is inactivated by more than 85% (Fig. 2). Extrapolation of the linear portion to zero activity indicates that the incorporation of approx. 6 mol phenylglyoxal per mol enzyme protomer (i.e., the modification of 3 arginine/protomer) correlates with the complete inactivation of ribulosebisphosphate carboxylase (Fig. 2). It may be postulated, however, that the amount of total reagent incorporation at any given time represents a composite of the modification of arginine residues whose reaction is unrelated to loss in activity or the presence of protective ligands and those groups that are essential for activity and whose modification is affected by ligands. Additional insight into the number of essential arginine residues involved at the active site can thus be obtained by comparing the amount of ^{14}C-incorporation and the percent carboxylase activity of samples treated with phenylglyoxal for the same length of time in the absence or presence of ribulose-P_2 or sedoheptulose-P_2 (Table IV). Assuming the difference in reagent incorporation per protomer is linearly related to the difference in activity between the inactivated and protected samples, the extrapolations indicate that complete protection against inactivation correlates with the shielding of 1—2 (1.27 ± 0.25) arginine residues per protomer (Table IV). This value compares favorably with similar, but less quantitative determinations based on amino acid analyses of the modified and substrate-protected enzymes (Tables I and III). As in the inactivation experiments (Table II), 6-*P*-gluconate has little effect on [^{14}C]phenylglyoxal incorporation (Table IV).

Preincubation of the tobacco carboxylase with the competitive inhibitor sedoheptulose-P_2 prevents the incorporation of about 2 mol [^{14}C]phenylglyoxal per mol enzyme protomer (i.e., the modification of 1 arginine/protomer) following a 45 min treatment with the reagent (Table IV). Samples of the carboxylase that had been treated with [^{14}C]phenylglyoxal (in either the absence or presence of sedoheptulose-P_2) for 45 min were dissociated and the large and small subunits separated by Sephadex G-100 gel filtration in the presence of SDS (Fig. 3, inset). As seen in the radioactivity profiles obtained by



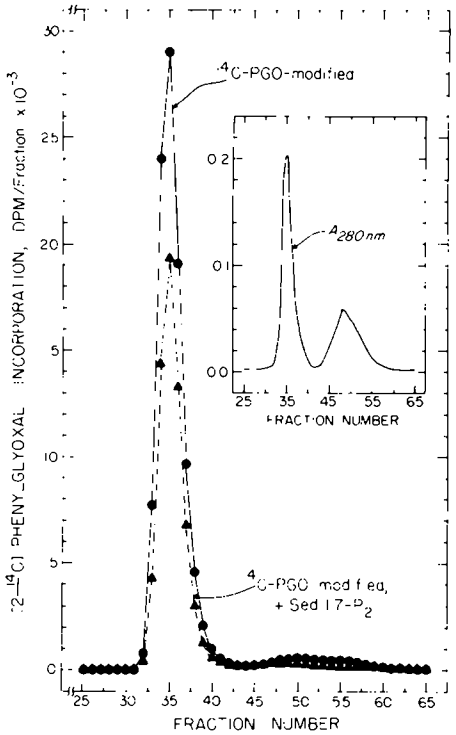


Fig 2 Correlation of [2-^{14}C]phenylglyoxal incorporation with tobacco ribulosebisphosphate carboxylase inactivation For details, see Extp Nos I (▲), II (■) and III (●) in Table IV PGO, phenylglyoxal

Fig 3 Sephadex G-100 gel filtration of [^{14}C]phenylglyoxal-modified tobacco ribulosebisphosphate carboxylase in the presence of 0 5% SDS at pH 8 6. Modification of 8.4 μM enzyme by 5 3 mM [2-^{14}C]phenylglyoxal (PGO) (1 25 Ci/mol) was carried out at 30°C in 50 mM Bicine buffer (pH 8 0) in the absence (●) or presence (▲) of 2 mM sedoheptulose-P_2 (Sed 1,7-P_2) The reaction was terminated after 45 min by chromatography through Sephadex G-25 and the ^{14}C-protein eluant processed as described in Materials and Methods The inset shows the profile of absorbance at 280 nm from the Sephadex G-100 column The large and small subunit peak fractions were numbers 35 and 48, respectively The large subunit was eluted with the excluded volume

counting aliquots of each fraction (Fig. 3), phenylglyoxal almost exclusively modifies residues in the large catalytic subunits of the protein even though the small subunits contribute 4—5 arginyl groups per mol enzyme protomer [23]. The apparent selectivity of this α-dicarbonyl for arginine residues in the catalytic subunits of ribulosebisphosphate carboxylase is likely due to the hydrophobic nature of this subunit type. These observations, however, must be somewhat qualified in that some lability of the [^{14}C]phenylglyoxal-protein adduct is observed during the 6 h period required to dissociate the protein and separate the subunits at alkaline pH (cf., Fig. 1). Preincubation of the protein with sedoheptulose-P_2 (Fig. 3), ribulose-P_2 or the transition-state analogue 2-carboxypentitol-1,5-P_2 (plus Mg^{2+}) markedly reduces the incorporation of [^{14}C]phenylglyoxal into the catalytic subunits, consistent with the notion that these ligands protect against inactivation by shielding essential arginine residues in the active site region of the carboxylase.

Effect of arginyl modification on the regulatory properties of ribulosebisphosphate carboxylase. Arginine residues have been implicated in the anionic effector binding sites of several regulatory enzymes, including fructose-1,6-bisphosphatase [6], phospho*enol*pyruvate carboxylase [32], aspartate transcarbamylase [25,33], NAD-isocitrate dehydrogenase [34], and NAD(P)-glutamate dehydrogenase [35]. Since the activity of ribulosebisphosphate carboxylase at low pCO_2 is modulated by various phosphorylated effectors [10,11], the effect of butanedione and phenylglyoxal modification on the regulatory properties of this enzyme has also been examined. Inactivation of the tobacco enzyme by 20 mM butanedione (15—80% loss of activity) does not alter the response of the enzyme to various anionic effectors [16]. If the rate of α-dicarbonyl reaction with the residues involved in effector modulation is slower than the reaction rate of essential arginyl groups influencing activity, it would be impossible to detect any change in regulatory properties using this experimental protocol since enzymic activity is used to monitor effector modulation. However, when the essential arginine residues of the carboxylase are protected by ribulose-P_2, the response of the modified, but catalytically active protein (cf., Tables I, III, IV) to NADPH and 6-*P*-gluconate is still essentially identical to that of the control and phenylglyoxal- (Table V) or butanedione-inactivated samples. These results indicate that if anionic effectors modulate carboxylase-oxygenase activity by binding at specific regulatory site(s), arginine residues are either not involved in binding these phosphorylated ligands to the enzyme or such groups are also protected from modification, directly or indirectly, when ribulose-P_2 binds at the catalytic center.

Concluding remarks The results of the present study indicate that inactivation of crystalline tobacco ribulosebisphosphate carboxylase by arginine-selective α-dicarbonyls results from the modification of 1—2 essential arginyl groups within the large catalytic subunit, presumably at the active site. In contrast to the report by Schloss et al. [9], no evidence was obtained to suggest half-of-the-sites reactivity. Since both arginine modification and inactivation of the

TABLE V

EFFECT OF PHENYLGLYOXAL MODIFICATION OF TOBACCO RIBULOSEBISPHOSPHATE CARBOXYLASE ON ACTIVATION BY ANIONIC EFFECTORS

Modification of 8 4 μM enzyme by 4 7 mM phenylglyoxal (PGO) was carried out at 30°C in 50 mM Bicine buffer (pH 8 0) in the absence or presence of 1 mM ribulose-P_2 (Rbu-P_2) After 50 and 145 min, aliquots were passed through a Sephadex G-25 column and the protein eluant was immediately assayed for carboxylase activity in the absence or presence of 0 1 mM 6-*P*-gluconate or 0 4 mM NADPH Control activity was 24 nmol $H^{14}CO_3^-$ fixed/min per mg protein

Enzyme	Carboxylase activity		
	% of control	+/— 6-*P*-gluconate	+/— NADPH
Control	100	3 48	2 80
PGO-modified	23	2 46	2 09
	<1	—	—
Rbu-P_2-protected	103	2 82	2 20
	70	2 85	2 03

higher plant-type carboxylase isolated from tobacco leaves and the procaryote *T intermedius* are decreased by the substrate ribulose-P_2 or the diphosphorylated competitive inhibitor sedoheptulose-P_2, these residues likely serve as binding sites for negatively charged phosphate groups of the non-gaseous substrate.

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