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CYANATE MODIFICATION OF ESSENTIAL LYSYL RESIDUES IN THE CATALYTIC SUBUNIT OF TOBACCO RIBULOSEBISPHOSPHATE CARBOXYLASE *

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Summary

Crystalline ribulose-1,5-bisphosphate carboxylase (3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39) isolated from tobacco (*Nicotiana tabacum* L.) leaf homogenates is irreversibly inactivated by incubation with potassium cyanate at pH 7.4. The rate of inactivation is pseudo first-order and linearly dependent on reagent concentration. In the presence of ribulosebisphosphate or high levels of CO_2 and Mg^{2+} the rate constant for inactivation is reduced, suggesting that chemical modification occurs in the active site region of the enzyme. In contrast, neither the effector NADPH nor the activator Mg^{2+} alone significantly affect the rate of inactivation by cyanate; however, NADPH markedly enhances the protective effect of CO_2 and Mg^{2+}. Incubation of the carboxylase with potassium [^{14}C]cyanate in the absence or presence of ribulosebisphosphate revealed that the substrate specifically reduces cyanate incorporation into the large catalytic subunits of the enzyme. Analysis of acid hydrolysates of the radioactive carboxylase indicated that the reagent carbamylates both NH_2-terminal groups and lysyl residues in the large and small subunits. Comparison of the substrate-protected enzyme with the inactivated carboxylase revealed that ribulosebisphosphate preferentially reduces lysyl modification within the large subunit. The data here presented indicate that inactivation of ribulosebisphosphate carboxylase by cyanate or its reactive tautomer, isocyanic acid, results from the modification of lysyl residues within the catalytic subunit, presumably at the activator and substrate CO_2 binding sites on the enzyme.

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Abbreviations: Rbu-P_2, ribulose 1,5-bisphosphate; Nbs_2, 5,5′-dithiobis-(2-nitrobenzoate); Rbu-5-*P*, ribulose 5-phosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Introduction

Ribulose-1,5-bisphosphate carboxylase (3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39) catalyzes the initial reaction in the photosynthetic carbon reduction cycle, the carboxylation of Rbu-P_2 to yield two molecules of 3-*P*-glycerate. The enzyme also functions as an internal monooxygenase, catalyzing the oxygenation of Rbu-P_2 to yield 3-*P*-glycerate and 2-*P*-glycolate, the precursor of the photorespiratory substrate, glycolic acid. The enzyme has been obtained in a highly purified crystalline state from tobacco leaves and is a huge protein ($s^0_{20,w} = 18.3$S, $M_r \approx 550\,000$) composed of eight 68.5-kdalton protomers, each consisting of one large (56-kdalton) catalytic subunit and one small (12.5-kdalton) non-catalytic subunit [1,2].

Since regulation of the ratio of Rbu-P_2 carboxylase activity to Rbu-P_2 oxygenase activity is believed to be equivalent to regulation of photosynthetic CO_2 fixation versus photorespiration [3], information pertaining to the over-all reactions catalyzed by this bifunctional enzyme has important implications with respect to agricultural productivity. Although the reaction mechanisms for carboxylation and oxygenation have been partially elucidated [4], the functional groups of the enzyme essential for catalysis have not been characterized thoroughly. Earlier studies with chemical modifiers suggested the essentiality of sulfhydryl groups [5]. However, it is uncertain whether -SH groups are involved in the catalytic process or in the maintenance of the native structural organization of the enzyme molecule [6,7]. More recently, chemical modification studies with the affinity labels 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate [8,9] and *N*-bromoacetylethanolamine phosphate [10] and with pyridoxal 5′-phosphate [11,12] have suggested the presence of essential lysyl residues in bacterial and higher plant Rbu-P_2 carboxylases. In addition, Lorimer et al. [13] presented evidence from kinetic studies which suggests that primary amino groups are involved in the reversible activation of Rbu-P_2 carboxylase-oxygenase by CO_2 and Mg^{2+}. According to their view, activation results from CO_2 combining with an uncharged amino group to form a carbamate, followed by rapid reaction with Mg^{2+} to form a ternary complex of enzyme · CO_2 · Mg which is then activated for carboxylation or oxygenation of Rbu-P_2.

Cyanate has been shown to irreversibly inhibit a variety of enzymes under neutral or weakly alkaline conditions by carbamylating the ϵ-amino group of lysyl residues [14—16]. In addition, it has been suggested that isocyanic acid (HN=C=O), the reactive tautomer of cyanate, is both a structural and electronic analogue of O=C=O [14,17,18]. We reasoned that cyanate might be a useful chemical modifier for further investigating the role of lysyl residues in Rbu-P_2 carboxylase, an enzyme for which CO_2 is both an activator and substrate. This study reports the effect of using potassium cyanate to specifically react with amino acid residues of tobacco Rbu-P_2 carboxylase. The results indicate that lysyl groups in the large catalytic subunits of the protein are essential for enzymic activity.

Materials and Methods

Materials. Potassium cyanate (reagent grade) was purchased from Fisher Scientific Co., potassium [^{14}C]cyanate (51 Ci/mol) from Amersham/Searle, and

L-homocitrulline from U.S. Biochemical Corp. Ellman's reagent (Nbs_2) and the sodium salts of Rbu-P_2, Rbu-5-P and NADPH were obtained from Sigma Chemical Co. and $NaH^{14}CO_3$ from New England Nuclear. Sephadex G-100 and prepacked columns of Sephadex G-25M (9.1 ml bed volume) were purchased from Pharmacia Fine Chemicals and sodium dodecyl sulfate (SDS) and Dowex 50W-X4 (200—400 mesh) from Bio-Rad Labs.

Enzyme preparation. Crystalline Rbu-P_2 carboxylase was obtained from tobacco (*Nicotiana tabacum* L. cv. Xanthi) leaf homogenates and recrystallized twice as described previously [19]. The thrice-crystallized protein exhibited two major bands in polyacrylamide gel electrophoresis in the presence of 0.1% (w/v) SDS (see Fig. 4, inset), corresponding to the 56- and 12.5-kdalton subunits of the enzyme. The protein crystals were dissolved in 50 mM Hepes, 0.1 M NaCl, pH 7.4, to yield a final concentration of approx. 15 mg/ml, and the resultant enzyme solution was heat reactivated for 20 min at 50°C [20].

Enzyme activity and protein determination. Rbu-P_2 carboxylase activity was determined by $^{14}CO_2$ incorporation at 30°C in the presence of $NaH^{14}CO_3$ and Rbu-P_2 [21]. The complete reaction mixture contained 0.1 M Tris, 10 mM $MgCl_2$, 0.1 mM Na_2EDTA (entire solution adjusted with NaOH to pH 7.9), 0.6 mM Rbu-P_2, 20 mM $NaH^{14}CO_3$ (0.25 Ci/mol), and 5—20 μg of protein in a final volume of 1.0 ml. Protein concentration was estimated by multiplying A_{280nm} in a cuvette of 1 cm light path by the factor 0.7 mg/ml [22]. The specific activity of the crystalline carboxylase preparations ranged from 300 to 500 nmol/min per mg protein. The molar concentration of tobacco Rbu-P_2 carboxylase was calculated using a molecular weight of 550 000 [23].

Inactivation of Rbu-P_2 carboxylase by cyanate. The cyanate inactivation was routinely studied by adding 0.5 ml of freshly prepared KNCO stock solution (adjusted to pH 7.4 in 50 mM Hepes buffer) to 0.3 ml of protein solution which had been preincubated for 15 min at 30°C in the absence or presence of various ligands. The final 0.8 ml solution contained 50 mM Hepes, 25 mM NaCl, 6—12 μM enzyme, various ligands, and KNCO at pH 7.4 as specified in the figure and table legends. Controls were treated identically except that KNCO was replaced with an equimolar concentration of KCl. After the specified times at 30°C, 50-μl aliquots were removed, diluted with 3 ml of 40 mM Tris, 5 mM NaCl, and 0.5 mM Na_2EDTA, pH 7.7, and 0.1-ml aliquots assayed for carboxylase activity. Alternatively, at the appropriate times the inactivation reaction was terminated by passing a portion of the reaction mixture through a small column of Sephadex G-25M equilibrated with 50 mM Hepes, 0.1 M NaCl, pH 7.7, and aliquots of the eluant assayed for activity. In most of the inactivation experiments a final concentration of 0.1 M KNCO was used. However, in the studies of the rate of inactivation as a function of cyanate concentration, the KNCO concentration ranged from 0.05 to 0.225 M. The ionic strength was maintained at a constant value by adding KCl.

Carbamylation of Rbu-P_2 carboxylase by cyanate. The carbamylation of Rbu-P_2 carboxylase was studied exactly as described above for inactivation except that unlabeled potassium cyanate was replaced with 0.1 M $KN^{14}CO$ (0.34 Ci/mol). After the specified times at 30°C, 50-μl aliquots (approx. 170 μg of protein) of the reaction mixture were transferred into 5 ml of ice-cold 5% (w/v) trichloroacetic acid to terminate the reaction. The precipitated samples

were filtered onto Millipore filters (0.45 μm pore, 25 mm diameter). The filters were washed eight times with 5-ml aliquots of cold 5% trichloroacetic acid, placed in 12 ml of Handifluor liquid scintillation counting solution (Mallinckrodt) and dpm were determined. Blanks were treated identically except that the protein solution was omitted from the reaction mixture. From the measured specific radioactivity of the [^{14}C]cyanate solution in the presence of a washed filter, the mol of cyanate incorporated per mol of enzyme protomer was calculated.

Labeling of enzyme for subunit studies and amino acid analyses. Labeled Rbu-P_2 carboxylase was prepared by 60 min incubation of the protein (approx. 7 μM) with 0.1 M KN^{14}CO (0.8 Ci/mol) at pH 7.4 and 30°C in the absence or presence of 5 mM Rbu-P_2. The reaction was terminated by adding an equal volume of ice-cold 10% trichloroacetic acid, and the precipitate was collected by centrifugation (1800 $\times g$) for 10 min at 4°C. The pellet was washed seven times with 2-ml portions of cold 5% trichloroacetic acid and once with 2 ml of cold 80% (v/v) acetone, and finally dried at 37°C.

Subunit isolation. The [^{14}C]cyanate-labeled protein precipitate (approx. 3 mg) was dissolved in 1.5 ml of 50 mM Tris buffer containing 1% (w/v) SDS and 0.14 M 2-mercaptoethanol at pH 9.0 and incubated for 3 h at 37°C under N_2, followed by an additional 2 min in a boiling water bath. The dissociated subunits were then separated at 25°C by chromatography on a 1.6 $\times$ 50 cm column of Sephadex G-100 equilibrated with 50 mM Tris buffer containing 0.5% SDS and 0.1 mM Na_2EDTA at pH 8.6 [24]. The subunits were eluted with the same buffer system at a flow rate of 20 ml/h and 1.5-ml fractions were collected. Aliquots of each fraction were analyzed for radioactivity and absorbance at 280 nm. After recovery of the protein from the pooled peak fractions by precipitation with 90% acetone for 60 h at 4°C, samples from each peak were subjected to SDS-polyacrylamide disc gel electrophoresis and amino acid analyses.

Electrophoresis was carried out at 25°C on pre-cast 12% polyacrylamide gels (Bio-Rad Labs.) in a 0.20 M Tris/0.20 M acetate buffer, pH 6.4, containing 0.1% SDS and 10 mM 2-mercaptoacetate essentially as described in Bio-Rad Bulletin No. 1038 (1976). Following electrophoresis, the gels were fixed with 40% 2-propanol/10% acetic acid, stained with 0.2% Coomassie Brilliant Blue R-250 in 10% 2-propanol/10% acetic acid, and destained in 2-propanol/acetic acid.

Sites of carbamylation of Rbu-P_2 carboxylase. For the determination of carbamylation at lysyl residues the [^{14}C]cyanate-labeled subunit or native protein precipitates were hydrolyzed in evacuated glass tubes at 110°C for 22 h with 6 M HCl, and the HCl was removed under reduced pressure. The acid hydrolysates were dissolved in 10% 2-propanol and subjected to two-dimensional chromatography on 20 $\times$ 20 cm pre-coated plates of Cellulose MN 300 (0.25 mm, Brinkman Instruments, Inc.) exactly as described by Haworth and Heathcote [25]. Radioactive products were located by radioautography (total exposure of at least $2.3 \cdot 10^7$ dpm) using Kodak X-Omat R film. Radioactive homocitrulline, the cyanate derivative of lysine, was identified by cochromatography with authentic L-homocitrulline. For the determination of carbamylation at NH_2-terminal residues, the [^{14}C]cyanate-labeled protein precipitates were

dissolved in 50% acetic acid and heated under vacuum with an equal volume of concentrated HCl for 1 h in a boiling water bath [26]. By this procedure, the carbamylated NH_2-terminal residues are cyclized to hydantoins. The subsequent separation of the hydantoin fraction from amino acids and peptides by cation-exchange column chromatography was performed as described by Stark [26].

Results

Inactivation of Rbu-P_2 carboxylase by cyanate

Cyanate is a relatively non-specific chemical modifier which has been found under various conditions to react with amino, sulfhydryl, carboxylic, phenolic hydroxyl, and imidazole groups of proteins [27]. However, only the products of reactions with amino groups, homocitrulline and *N*-α-amino carbamyl derivatives, are stable at neutral or weakly alkaline pH in absence of reagent [27]. Rbu-P_2 carboxylase activity was substantially inhibited by a 1 h preincubation of the enzyme with 0.1 M KNCO at pH 7.4 (Table I). The inhibition was observed following either gel filtration of the modified protein at pH 7.7 (Table I) or dilution (60-fold) with pH 7.7 Tris buffer, suggesting that the reaction which resulted in irreversible loss of activity involved only an amino group(s). This notion was supported by the observation that there was no irreversible modification of sulfhydryl groups on the inactivated enzyme (Table I).

A plot of log carboxylase activity versus time of cyanate treatment was linear, indicating its first-order dependence (Fig. 1). Since such plots were linear, pseudo first-order rate constants for inactivation (k_{obs}) were calculated by multiplying the slopes of the lines by 2.303. The inactivation of 7 μM Rbu-P_2 carboxylase by 0.1 M KNCO exhibited a pseudo first-order rate constant of 0.014 min^{-1} at pH 7.4 and 30°C. The rate of inactivation increased with increasing cyanate concentration at constant pH, ionic strength and enzyme concentration. The pseudo first-order rate constant followed a linear relationship with cyanate concentration up to 0.225 M, characteristic of an

TABLE I

ENZYMIC ACTIVITY AND SULFHYDRYL GROUP CONTENT OF Rbu-P_2 CARBOXYLASE FOLLOWING REACTION WITH CYANATE

The enzyme (7.1 μM) was incubated at 30°C with 0.1 M KCl (control) or KNCO, pH 7.4, for 1 h. When added, Rbu-P_2 was preincubated with the protein for 15 min prior to KCl or KNCO addition. The reaction was terminated by passage through a small column of Sephadex G-25 M (equilibrated at pH 7.7) and the eluant assayed for carboxylase activity and sulfhydryl content.

Treatment	Carboxylase activity (%)	-SH groups * (%)
Control	100	100
Plus 0.1 M KNCO	33	101
Control plus 5 mM Rbu-P_2	106	101
0.1 M KNCO plus 5 mM Rbu-P_2	104	105

* Determined by reaction with Nbs_2 at pH 7.7 (molar ratio of Nbs_2: protein was 1130 : 1) in the presence of 1% (w/v) SDS [20].

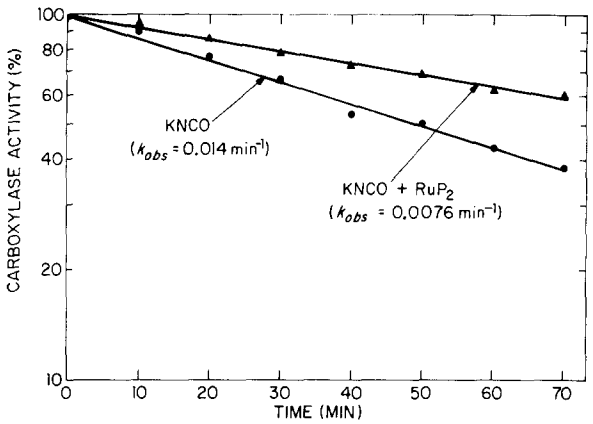

Fig. 1. Inactivation of 6.8 μM Rbu-P_2 carboxylase (54 μM protomeric units) by 0.1 M KNCO at pH 7.4 and 30°C, in the absence (●) or presence (▲) of 60 μM Rbu-P_2. After the specified times, 50-μl aliquots were removed, diluted with 3 ml of Tris buffer, pH 7.7, and aliquots assayed for carboxylase activity. The pseudo first-order rate constants for inactivation (k_{obs}) were calculated by multiplying the slopes of the computer-fitted lines by 2.303.

overall second-order reaction (Fig. 2). Thus, there was no evidence of a rate-limiting step involving non-covalent enzyme · cyanate complex formation prior to covalent modification.

Effect of various ligands on inactivation

The inactivation of Rbu-P_2 carboxylase by 0.1 M KNCO was almost completely prevented by preincubating the enzyme with high levels of the substrate, Rbu-P_2 (Table I). In experiments in which the molar ratio of Rbu-P_2 to protomeric unit was essentially unity, the substrate was still a very effective antagonist of cyanate inactivation, reducing k_{obs} by about 45% (Fig. 1). Of the non-substrate ligands examined, including Rbu-5-*P*, the activator Mg^{2+}, and the effector NADPH, only Rbu-5-*P* affected the rate of inactivation (Table II, lines 1, 2, 5, 7). However, on an equimolar basis, Rbu-P_2 was more than twice as effective as the monophosphate in reducing k_{obs} (lines 6 vs. 5). Mg^{2+}, in combination with Rbu-P_2, decreased the extent of substrate protection presumably by allowing the enzymic conversion of Rbu-P_2 to 3-*P*-glycerate in the presence of trace amounts of endogenous CO_2 (lines 1—4). When the enzyme was preincubated with 10 mM Mg^{2+} plus 10 mM bicarbonate (approx. 0.55 mM CO_2 at pH 7.4) there was little effect on k_{obs} (line 8). However, the rate of cyanate inactivation was reduced 35—55% by including the effector NADPH in the Mg^{2+}/HCO_3^- preincubation medium or increasing the bicarbonate concentration to 25 mM (lines 9—10) (in order to enhance the CO_2-Mg^{2+} activation process at pH 7.4 (see refs. 13 and 28).

Carbamylation of Rbu-P_2 carboxylase by cyanate

The time course of incorporation of [^{14}C]cyanate into Rbu-P_2 carboxylase is shown in Fig. 3. As in the inactivation experiments (Table II), the substrate Rbu-P_2 also reduced cyanate carbamylation of the protein (Fig. 3), whereas 2 mM NADPH or 10 mM HCO_3^-/Mg^{2+} were essentially without effect (data

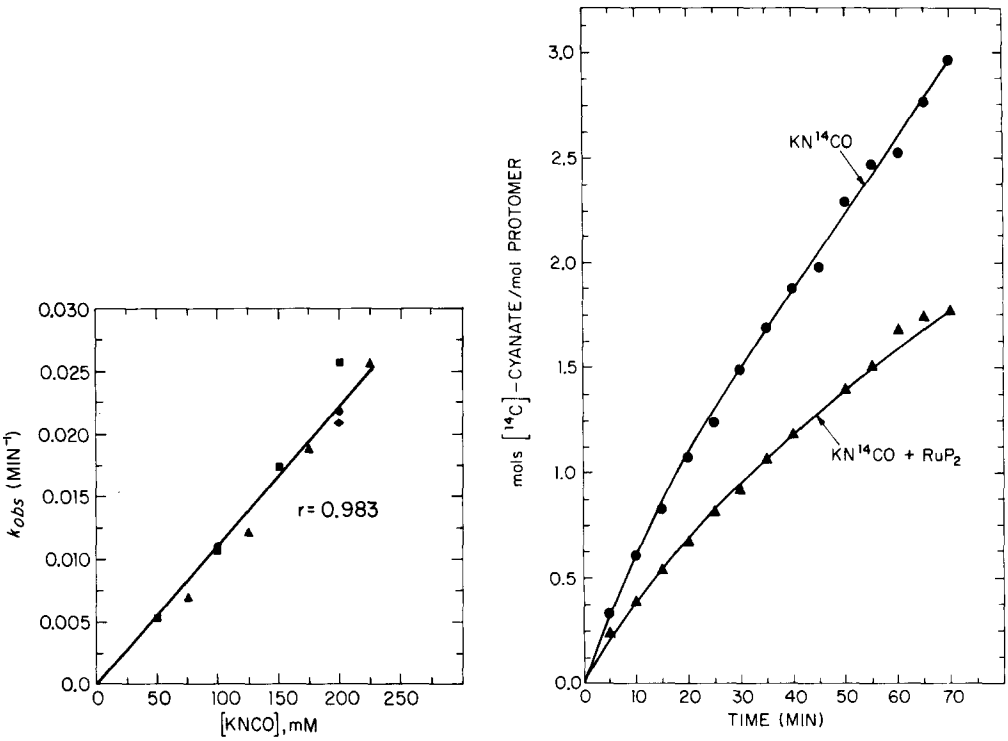

Fig. 2. Effect of cyanate concentration on the pseudo first-order rate constant for inactivation of 11.9 μM Rbu-P_2 carboxylase at pH 7.4 and 30°C. The pseudo first-order rate constants were determined under the conditions given in Fig. 1 in the absence of Rbu-P_2. The potassium cyanate concentration was varied, with KCl being added to maintain the ionic strength constant. Different symbols represent the results from separate experiments.

Fig. 3. Carbamylation of 6.1 μM Rbu-P_2 carboxylase by 0.1 M KN^{14}CO at pH 7.4 and 30°C, in the absence (●) or presence (▲) of 3 mM Rbu-P_2. After the specified times, 50-μl aliquots of the reaction mixture were transferred into 5 ml of ice-cold 5% trichloroacetic acid and processed as described in Materials and Methods.

TABLE II

EFFECT OF VARIOUS LIGANDS ON THE RATE OF INACTIVATION OF Rbu-P_2 CARBOXYLASE BY CYANATE

The enzyme (6.7 μM) was incubated at 30°C with 0.1 M KNCO, pH 7.4, for up to 70 min. When added, ligands (in 50 mM Hepes buffer, pH 7.4) were preincubated with the protein for 15 min prior to cyanate addition. The pseudo first-order rate constants were determined as described for Fig. 1.

	Ligand preincubated	k_{obs} (min^{-1} X 10^3)
1	None	14.8
2	5 mM Mg^{2+}	14.9
3	0.13 mM Rbu-P_2	6.7
4	5 mM Mg^{2+}/0.13 mM Rbu-P_2	13.2
5	2 mM Rbu-5-P	11.3
6	2 mM Rbu-P_2	5.5
7	2 mM NADPH	14.5
8	10 mM HCO_3^-/10 mM Mg^{2+}	13.1
9	2 mM NADPH plus 10 mM HCO_3^-/10 mM Mg^{2+}	6.8
10	25 mM HCO_3^-/10 mM Mg^{2+}	9.4

not shown). The relatively low level of incorporation (in either the absence or presence of Rbu-P_2) was indicative of a fairly specific modification, as there are 33 lysyl residues per tobacco carboxylase protomer [29].

Preincubation of the enzyme (6 μM) with 3 mM Rbu-P_2 prevented the modification of about one group per protomeric unit following a 1 h cyanate treatment at pH 7.4 (Fig. 3). Samples of the carboxylase that had been treated with 0.1 M $KN^{14}CO$ (in either the absence or presence of Rbu-P_2) for 1 h were dissociated and the large and small subunits separated by Sephadex G-100 gel filtration in the presence of 0.5% SDS (Fig. 4). The separated subunits were free of cross-contamination as judged by SDS gel electrophoresis (Fig. 4, inset). As seen in the radioactivity profiles obtained by counting aliquots of each fraction (Fig. 5), cyanate preferentially carbamylated residues of the large subunit, since the ratio of total ^{14}C incorporation into the large versus small subunits (approx. 6.3 to 1) exceeded the ratio of lysyl residues between the two subunits (3.1 to 1 [29]). More noteworthy, the substrate Rbu-P_2 specifically

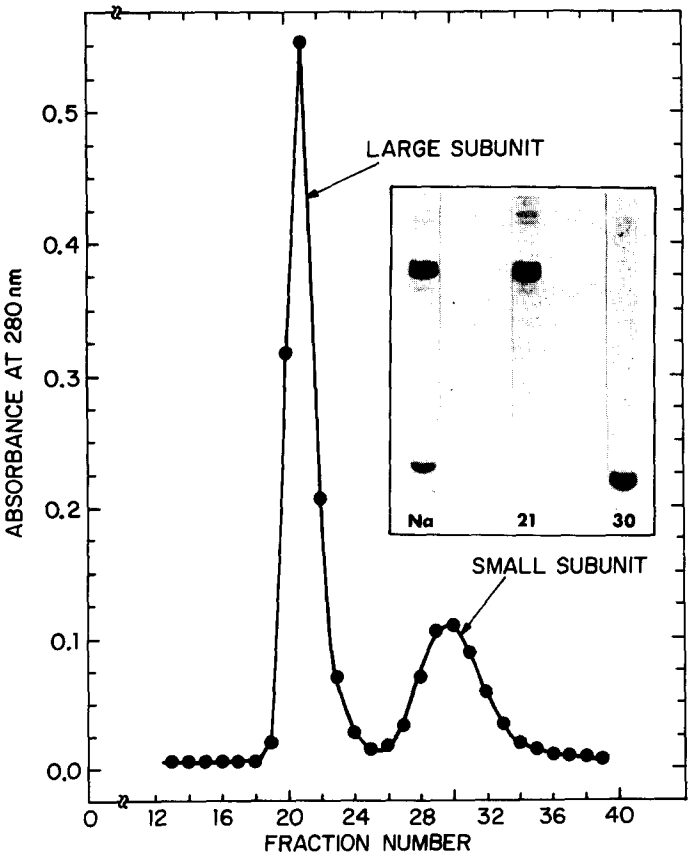

Fig. 4. Gel filtration of cyanate-modified Rbu-P_2 carboxylase (approx. 3 mg of protein) on Sephadex G-100 equilibrated with Tris buffer, pH 8.6, containing 0.5% SDS. The large subunit was eluted with the excluded volume. The inset shows SDS gels of the large (No. 21) and small (No. 30) subunit peak fractions and of the native cyanate-modified protein (Na).

reduced the amount of cyanate incorporation into the large catalytic subunit (Fig. 5).

Identification of amino acid residues modified by cyanate

Cyanate is capable of reacting with several functional groups in proteins, but only amino groups yield stable derivatives. Other functional groups yield unstable carbamylated products which readily decompose to the original amino acids upon removal of excess cyanate at neutral or weakly alkaline pH [27]. The observation that the cyanate-modified carboxylase remained inactivated following gel filtration (Table I) or dilution (Fig. 1) at pH 7.7 suggested that the carbamylation of amino groups was responsible for the observed inactivation.

Radioactively labeled Rbu-P_2 carboxylase was prepared by a 1 h incubation with 0.1 M $KN^{14}CO$, pH 7.4, in either the absence or presence of 5 mM Rbu-P_2. The washed protein precipitate was either directly hydrolyzed to yield a free amino acid or hydantoin fraction (see Materials and Methods section) or first dissociated and separated into subunits prior to acid hydrolysis. Based on ^{14}C incorporation into the hydantoin and free amino acid fractions, cyanate carbamylated both NH_2-terminal groups and non-terminal residues of the large and small subunits in either the absence or presence of Rbu-P_2. When the free amino acid fractions were resolved by two-dimensional thin-layer chromatog-

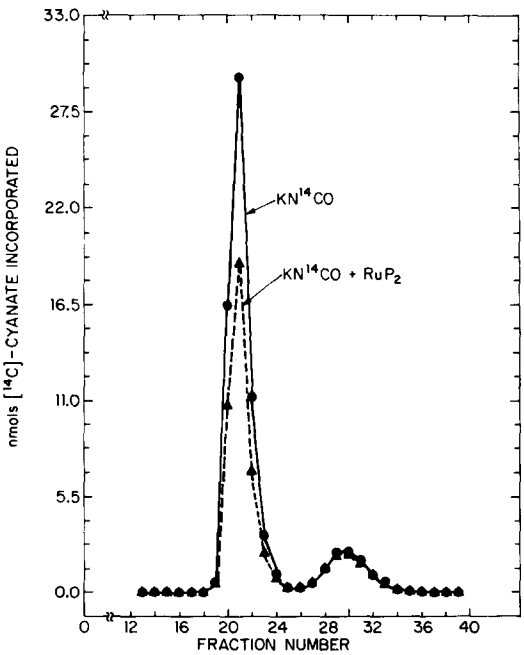

Fig. 5. Sephadex G-100 gel filtration of carbamylated Rbu-P_2 carboxylase in the presence of 0.5% SDS. The enzyme (6.4 μM) was incubated at 30°C with 0.1 M $KN^{14}CO$, pH 7.4, in the absence (●) or presence (▲) of 5 mM Rbu-P_2. The reaction was terminated after 1 h by the addition of an equal volume of ice-cold 10% trichloroacetic acid and the precipitated protein processed as described in Materials and Methods. The data points for any given fraction are normalized with respect to A_{280nm}.

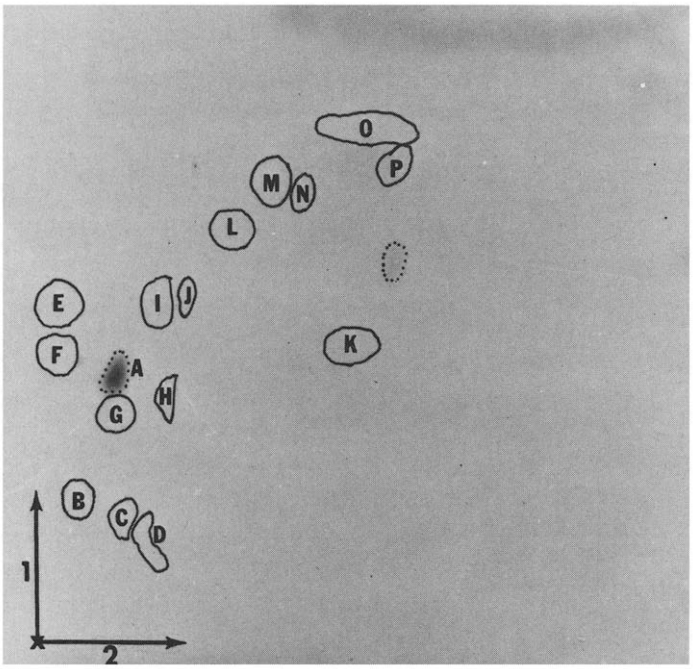

Fig. 6. Radioautogram of a two-dimensional thin-layer chromatogram of the acid hydrolysate of [^{14}C]-cyanate-inactivated Rbu-P_2 carboxylase. The enzyme (6.9 μM) was incubated at 30°C with 0.1 M KN^{14}CO, pH 7.4, for 1 h. The acid hydrolysate, prepared as described in Materials and Methods was subjected to two-dimensional thin-layer chromatography, developed first with 2-propanol/methyl ethyl ketone/1 M HCl (60 : 15 : 25, v/v) and then with *tert*-butyl alcohol/methyl ethyl ketone/acetone/methanol/water/ammonium hydroxide (40 : 20 : 20 : 01 : 14 : 05, v/v) as described by Haworth and Heathcote [25]. The acid hydrolysates of the [^{14}C]cyanate-labeled large and small subunits yielded identical radioautograms, as did the substrate-protected carboxylase. The position of the amino acids (encircled areas) was ascertained by reaction with ninhydrin. A, authentic L-homocitrulline; B, arginine; C, lysine; D, histidine; E, glutamic acid; F, aspartic acid; G, glycine; H, serine; I, alanine; J, proline; K, threonine; L, tyrosine; M, valine; N, methionine; O, leucine/isoleucine; P, phenylalanine; x, origin. The dotted lines represent areas of radioactivity. The minor, ninhydrin-negative radioactive spot corresponds to a degradation product of homocitrulline [14].

raphy the major radioactive spot cochromatographed in every case with authentic L-homocitrulline (Fig. 6), the cyanate derivative of lysine. When the substrate-protected enzyme was compared with the inactivated carboxylase, it was noted that Rbu-P_2 preferentially reduced cyanate modification of lysyl residues such that the ratio of total ^{14}C incorporation into the NH_2-terminal versus homocitrulline fractions increased by about 55%, from 0.43 : 1 in the absence of Rbu-P_2 to 0.67 : 1 in its presence (data not shown).

Discussion

These experiments indicate that tobacco leaf Rbu-P_2 carboxylase is irreversibly inactivated by covalent reaction of cyanate with primary amino groups. Modification resulting in the irreversible loss of activity does not occur within the binding site for either Mg^{2+} or NADPH since neither the activator nor the

effector protects against inactivation. In contrast, almost complete protection is provided by high levels of the substrate Rbu-P_2 suggesting that inactivation results from the carbamylation of amino groups within the binding domain for Rbu-P_2. This notion is supported by the observation that the substrate preferentially reduces lysyl modification within the large catalytic subunit. However, as is the case in most chemical modification studies, the possibility that binding of Rbu-P_2 at the catalytic center induces a conformational change which results indirectly in a reduced rate of lysyl modification at a distant site on the protein cannot be excluded. If such a protective conformational change takes place, it must be restricted to the catalytic subunit since Rbu-P_2 specifically reduces large subunit carbamylation. Thus, the most straightforward interpretation of our results suggests that essential lysyl residues are located within the binding domain for Rbu-P_2 on the large catalytic subunit, presumably at the active site of the carboxylase.

Lysyl residues have previously been implicated in the active site region of bacterial and higher plant Rbu-P_2 carboxylases based on studies of modification of the enzyme by pyridoxal-5′-*P* [11,12] and phosphorylated affinity labels [8—10]. A common feature of these previous chemical modification studies with the present experiments is that in every case the substrate Rbu-P_2 protects the enzyme from inactivation. In addition, the present cyanate study (Fig. 5) and the previous experiments with the affinity label 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate [8,9] indicate that when the substrate protects against inactivation, it does so by specifically reducing the modification of lysyl residues contained within the large catalytic subunit. Paech et al. [12] have recently proposed that a primary amino group (presumably an ϵ-amino group of a lysyl residue) is located at the catalytic center of the enzyme and is presumably the substrate CO_2 binding site. According to their view, Rbu-P_2 protects this amino group from chemical modification by reacting via its C-2 carbonyl function to form an aminocarbinol. However, the assumption that Rbu-P_2 directly reacts with the amino group at the CO_2 binding site is not entirely consistent with several observations. Firstly, based on this assumption one would predict that Rbu-5-*P* might also effectively protect this amino group from modification since it is very similar to Rbu-P_2. This has not been the case either in the present experiments (Table II) or in a previous study with pyridoxal-5′-*P* [11]. Secondly, if Rbu-P_2 protects this essential amino group by forming an aminocarbinol, this reaction must be readily reversible since preincubating the enzyme with high levels of Rbu-P_2 does not itself result in an irreversible loss of activity (see Table I and ref. 11). Perhaps a simpler interpretation is that binding of the substrate Rbu-P_2 to the active site sterically interferes, either directly or indirectly via an induced conformational change [30], with the access of chemical modifiers to essential amino groups within the large catalytic subunit.

Although substrate protection is a common feature of all the amino group modification studies with Rbu-P_2 carboxylase, the effects of various other ligands on the rate of inactivation are quite contradictory. For example, Mg^{2+} (5 mM) has no effect on the irreversible inactivation by cyanate (Table II) or 3-bromo-1,4-dihydroxy-2-butanone-1,4-bisphosphate [31], yet the activator stimulates the rate of modification by the affinity label *N*-bromoacetylethanol-

amine phosphate [10]. Similarly, high levels of Mg^{2+} (10 mM) in combination with bicarbonate (up to 0.15 M) have essentially no effect on the reversible modification of Rbu-P_2 carboxylase by pyridoxal-5'-P [12], whereas preincubation of the enzyme with 10 mM Mg^{2+} and either 25 mM HCO_3^- or 10 mM HCO_3^- plus NADPH reduces inactivation by cyanate (Table II). Two primary amino groups per protomeric unit of carboxylase have been proposed to be essential for enzymic activity [8,12]. One presumably is involved in the CO_2-Mg activation reaction and the other in binding substrate CO_2 to the active site. Isocyanic acid, the reactive tautomer of cyanate, probably reacts with both the activator and substrate CO_2 binding sites on the enzyme since it is presumably an electronic and structural analogue of carbon dioxide [14,17,18]. In contrast, phosphorylated chemical modifiers probably react preferentially with groups within the binding domain for the phosphorylated substrate Rbu-P_2. The observation that the effector NADPH markedly enhances the protective effect of Mg^{2+} and HCO_3^- against cyanate inactivation is consistent with this modifier reacting, in part, with the activator CO_2 binding site. NADPH has been reported to decrease the apparent dissociation constant for CO_2 in the activation reaction [28], possibly by altering the pK of the amino group with which activator CO_2 reacts [13]. However, if cyanate reacts both with activator and substrate CO_2 binding sites, these groups must be located within the large catalytic subunit and protected, either directly or indirectly, by Rbu-P_2 binding, since only residues in the large subunits of the substrate-protected enzyme are specifically protected from carbamylation (Fig. 5). Thus a major question to be resolved is whether the essential amino groups modified by cyanate and the phosphorylated chemical modifiers correspond to the same lysyl residue(s) within the large catalytic subunit. Such information would provide valuable insight into the interpretation of the seemingly contradictory ligand effects on the reaction of amino groups of Rbu-P_2 carboxylase with chemical modifiers.

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