THE ABSENCE OF TIGHTLY BOUND COPPER, IRON, AND FLAVIN NUCLEOTIDE IN CRYSTALLINE RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE-OXYGENASE FROM TOBACCO*

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Summary

Crystalline ribulose 1,5-bisphosphate carboxylase-oxygenase (EC 4.1.1.39) was isolated from tobacco (Nicotiana tabacum L.) leaf homogenates and analyzed for several characteristic oxygenase prosthetic groups. Analyses by atomic absorption and emission spectroscopy and neutron activation indicated that the crystalline protein contains less than 0.2 g-atoms of tightly bound copper or iron per mole (550,000 g) of enzyme. In addition, the absorption and fluorescence spectra of concentrated solutions of the crystalline protein gave no indication of the presence of a flavin nucleotide. Thus, the enzymatic oxygenation of ribulose 1,5-bisphosphate to yield P-glycolate, which is believed to comprise the initial reaction in the photorespiratory metabolism of higher plants, appears not to involve these cofactors in the catalytic mechanism.

Introduction

Ribulose 1,5-bisphosphate carboxylase catalyzes the initial reaction in the photosynthetic carbon reduction cycle, the carboxylation of Ru-P_2 to yield two moles of 3-PGA (1). More recent work with purified preparations of the carboxylase isolated from prokaryotic and eukaryotic organisms has demonstrated that the enzyme also functions as an oxygenase,

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Abbreviations: Ru-P_2, ribulose 1,5-bisphosphate; 3-PGA, 3-phosphoglycerate; cryst, crystalline.

catalyzing the oxygenation of Ru-P_2 to yield 3-PGA and P-glycolate (2-8), the presumed precursor of the photorespiratory substrate, glycolic acid. CO_2 and O_2 apparently compete for Ru-P_2 at the same or at adjacent sites on the enzyme, since O_2 is a competitive inhibitor with respect to CO_2 in the carboxylase reaction and CO_2 is a competitive inhibitor with respect to O_2 in the oxygenase reaction (5, 7-10). It has been suggested that this competition between CO_2 and O_2 for Ru-P_2 at the carboxylase determines the relative rates of photosynthesis and photorespiration in leaves of C_3 plants, with high CO_2 or low O_2 favoring carboxylation and therefore photosynthesis, while low CO_2 or high O_2 favor oxygenation and therefore glycolate synthesis and photorespiration (for review, see Ref. 11).

All oxygenases studied to date contain at least one additional component which is presumably involved in the catalytic mechanism besides the protein moiety. Iron has been reported to be a common cofactor of the dioxygenases, whereas in the case of the monooxygenases, the nature of the prosthetic group is more diverse, including such components as copper, iron, and flavin nucleotide (12, 13). Wishnick et al. (14, 15) have reported that homogenous, non-crystalline preparations of Ru-P_2 carboxylase isolated from spinach contain 1 g-atom of tightly bound copper (II) per mole of enzyme. However, Lorimer et al. (4) have recently questioned these findings, having observed that purified preparations of non-crystalline spinach Ru-P_2 carboxylase-oxygenase contain less than 0.2 g-atoms of copper per mole of enzyme.

In studies related to the mechanism of the Ru-P_2 oxygenase reaction, we have reinvestigated the metal content of this bi-functional enzyme using thrice-crystallized preparations of Ru-P_2 carboxylase-oxygenase isolated from tobacco. The results from this study are the subject of this communication.

Materials and Methods

Reagents. All biochemicals were purchased from Sigma Chemical Co. [^{14}C]$NaHCO_3$ was obtained from New England Nuclear and analyzed He/O_2 mixtures were purchased from Air Products and Chemicals, Inc.

Plant material. Plants of Nicotiana tabacum L., var. Coker 219, were grown in a growth chamber with an 18-hr day at 24°C and a 6-hr night at 19°C. Light of approx 60 klux at the upper leaves was provided by incandescent and cool-white fluorescent lamps. Only the younger, fully-expanded leaves from 4-month-old plants were used.

Isolation and crystallization of Ru-P_2 carboxylase. Crystalline Ru-P_2 carboxylase was obtained from tobacco leaf homogenates and recrystallized essentially as described by Chan et al. (16) except that the protein crystals were dissolved in buffer D (0.025 M Tris-HCl, pH 7.4, 0.10 M NaCl, and 0.5 mM EDTA) plus the minimum amount of 1 M NaCl required to complete dissolution. In a typical isolation, 75 g of demidribbed tobacco leaves yielded approx 200 mg of thrice-crystallized, lyophilized Ru-P_2 carboxylase. The weight of the lyophilized crystals agreed within ±5% of the yield of cryst protein based on $A_{280\,nm}$ (see below).

To reduce contamination of the cryst protein by extraneous metals during the isolation procedure, the following precautions were taken: (a) all magnetic stirring bars were washed with aqua regia and thoroughly rinsed with distilled water; (b) the dialysis tubing was boiled in 0.01 M EDTA and thoroughly rinsed; and (c) all glass centrifuge tubes were washed with concentrated H_2SO_4 and thoroughly rinsed.

Absorption spectrum and protein determination. The absorption spectrum (240-700 nm) and protein concentration of the dissolved crystals were determined in a Cary 15 spectrophotometer against buffer D. An absorbance of 1.0 at 280 nm was taken to represent 0.7 mg of protein/ml (17).

Enzyme assays. Unless noted otherwise, all enzyme assays were performed with thrice-crystallized, heat activated enzyme. Heat activation consisted of heating a 5 mg/ml solution of the protein for 20 min at 50°C as described in detail elsewhere (18).

Ru-P_2 carboxylase activity was determined by $^{14}CO_2$ incorporation at 25°C in the presence of $NaH^{14}CO_3$ and Ru-P_2. Unless noted otherwise, the reaction vessels contained 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 0.1 mM Na_2EDTA, 25.0 mM $NaH^{14}CO_3$ (0.3 Ci/mole), 0.4 mM Ru-P_2, and 20-25 μg of protein in a final volume of 1.0 ml. Vessels containing the buffer-salt solution and enzyme were sealed and repeatedly

evacuated and refilled with the appropriate He/O_2 mixture, followed by incubation for 10 min at 25°C in the presence of $NaH^{14}CO_3$. The reactions were initiated by injecting Ru-P_2 and terminated after 6 min by injecting 0.1 ml of 6 N acetic acid. Contents of the flasks were thoroughly mixed, aliquots dried at 90°C, and dpm determined by liquid scintillation spectroscopy.

Ru-P_2 oxygenase activity was determined by measuring P-glycolate formation at 25°C. Unless noted otherwise, the reaction vessels contained 50 mM Tris-HCl (pH 8.6), 10 mM $MgCl_2$, 0.1 mM Na_2EDTA, 0.6 mM Ru-P_2, and 100 µg of protein in a final volume of 1.0 ml. Vessels containing the buffer-salt solution and enzyme were sealed and repeatedly evacuated and refilled with the appropriate He/O_2 mixture, followed by preincubation for 10 min at 25°C. The reactions were initiated by injecting Ru-P_2 and terminated after 6 min by injecting 0.1 ml of 1 N HCl. P-glycolate produced was determined colorimetrically as described elsewhere (5). Blanks were treated in an identical manner except that enzyme was added following acidification. Recovery of P-glycolate internal standards was >90%.

Metal content. Thrice-crystallized, lyophilized preparations of Ru-P_2 carboxylase-oxygenase were routinely analyzed for metal content by atomic absorption and emission spectroscopy. For atomic absorption analyses, samples of the cryst, lyophilized protein weighing from 140 to 300 mg were ashed in porcelain crucibles at 500°C in a muffle furnace, dissolved in 1 N HNO_3, and brought to 25.0 ml with distilled water. The metal content of the solution was determined with a Jarrell-Ash model 810 dual channel, dual beam atomic absorption spectrophotometer with background correction. The recovery of copper and iron internal standards (100-mg samples of crystallized bovine serum albumin dissolved in 2.0 ml of 0.10 mM $CuSO_4$ or $FeSO_4$, and lyophilized) was greater than 85%.

Four 15- to 25-mg samples of thrice-crystallized, lyophilized Ru-P_2 carboxylase-oxygenase were also analyzed for copper content by a nondestructive neutron activation analysis performed at the Phoenix Memorial Laboratory, The University of Michigan (Ann Arbor).

Results and Discussion

Purity and other properties of crystalline Ru-P_2 carboxylase. After each of the three crystallization steps, the preparations of Ru-P_2 carboxylase were examined for purity by analytical

ultracentrifugation and absorption spectroscopy. The sedimentation patterns of once-, twice-, and thrice-crystallized protein all revealed a single symmetrical component with no evidence of inhomogeneity. The $\underline{s}^{0}_{20,w}$ value of the thrice-crystallized enzyme was 18.3 S. The uv absorption spectrum of the dissolved protein after each stage of crystallization showed a maximum at 279 nm and minimum at 250 nm and an $\underline{A}_{280nm}/\underline{A}_{260nm}$ of 1.9 (Fig. 1), which is characteristic of pure Ru-P_2 carboxylase (19,20). In addition, solutions of the cryst enzyme in protein concentrations up to 5.5 mg/ml showed no absorption maxima between 300 and 700 nm and little absorbance (<0.06 units) between 370-700 nm (Fig. 1), suggesting the absence of such

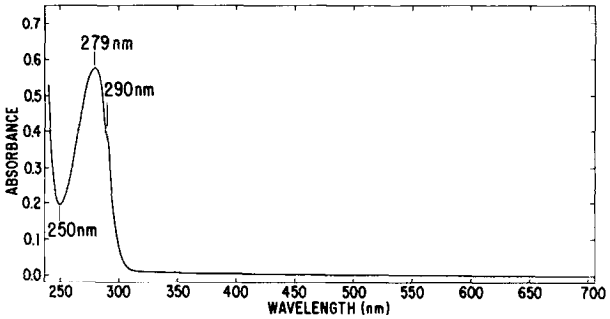

Fig. 1. Absorption spectrum of once-crystallized Ru-P_2 carboxylase. The protein concentration was 0.40 mg per ml buffer D.

prosthetic groups as FAD and FMN (21,22). Fluorescence spectra of 1 mg/ml solutions of the cryst protein determined at neutral and alkaline pH revealed only the presence of aromatic amino acids (data not shown), thus confirming the absence of flavin prosthetic groups (22).

As with the sedimentation patterns and absorption spectra, constant Ru-P_2 carboxylase specific activity was attained at the first crystallization. This observation differs from that previously reported with cryst prepar-

ations of the tobacco enzyme in which constant carboxylase specific activity was observed following the second crystallization step (17).

CO_2 fixation catalyzed by crystalline Ru-P_2 carboxylase. Thrice-crystallized, heat activated preparations of Ru-P_2 carboxylase had a specific activity of 320-540 nmoles HCO_3^- fixed min^{-1} mg $protein^{-1}$ when assayed under He at 25 mM $NaH^{14}CO_3$. The addition of 100 μM NaCN to the standard assay solution prior to initiation of the reaction with Ru-P_2 inhibited the carboxylase activity by about 40% (data not shown). Cyanide inhibition of Ru-P_2 carboxylase activity has previously been reported with the non-cryst, copper-free and copper-containing enzyme from spinach (15,23).

The effect of oxygen on CO_2 fixation catalyzed by cryst Ru-P_2 carboxylase is given in Table I. At 21% O_2 and 2.5 mM $NaH^{14}CO_3$, CO_2 uptake was inhibited by about 10%, and the inhibition increased with increasing O_2 tension. The inhibitory effect of O_2 was markedly reduced by increasing the level of bicarbonate (Table I), indicating that a sulf-

TABLE I. OXYGEN INHIBITION OF CO_2 FIXATION BY CRYSTALLINE Ru-P_2 CARBOXYLASE

Expt. No.	mM $NaH^{14}CO_3$	nmoles HCO_3^- fixed/min/mg protein 0% O_2	21% O_2	80% O_2	100% O_2	Percent inhibition
I	2.5	123	113			8
	25.0	416	415			0
II	2.5	94		65		31
	25.0	315		308		2
III	2.5	110			62	44
	25.0	352			330	6

hydryl oxidation was not responsible for the observed effect. These O_2 effects on the cryst carboxylase are similar to those previously reported with purified, non-cryst preparations of the bacterial (8), algal (7), and higher plant (5,9) enzyme.

P-glycolate formation catalyzed by crystalline Ru-P_2 carboxylase. Thrice-crystallized, heat activated preparations of Ru-P_2 carboxylase catalyzed the formation of P-glycolate from Ru-P_2 (Table II). Correlative studies using

TABLE II. OXYGENASE ACTIVITY OF CRYSTALLINE Ru-P_2 CARBOXYLASE

Expt. No.	Experimental conditions	nmoles P-glycolate/min/mg protein
I	21% O_2	28.6
	47% O_2	39.9
	100% O_2	56.2
II	21% O_2	26.1
	21% O_2, plus 4 μM $CuCl_2$	26.0
	21% O_2, plus 4 μM $FeCl_3$	25.2
	21% O_2, plus 100 μM NaCN	13.8

an O_2 electrode demonstrated that the cryst enzyme also catalyzed an Ru-P_2-dependent uptake of O_2 when assayed in the air-saturated condition (data not shown). P-glycolate formation was not stimulated by addition to the standard assay solution of a 20-fold molar excess of copper or iron to enzyme (Table II). As with the carboxylase activity, cyanide was also a potent inhibitor of the oxygenase reaction, 100 μM NaCN inhibiting the formation of P-glycolate by 47% (Table II). Cyanide inhibition of Ru-P_2 oxygenase activity has previously been reported with the non-cryst, copper-free enzyme from spinach (4).

Metal content of crystalline Ru-P_2 carboxylase.

Thrice-crystallized, lyophilized preparations of Ru-P_2 carboxylase-oxygenase were analyzed for copper, iron, and six other metals by atomic absorption and emission spectroscopy. The results of this metal survey, summarized in Table III, revealed that none of the metals evaluated were present in

TABLE III. METAL CONTENT OF CRYSTALLINE Ru-P_2 CARBOXYLASE

Metal	Metal content* g-atoms/mole carboxylase		
	AA**	ES$^{\delta}$	NA$^{\delta\delta}$
Cu	0.15 (7)	<0.32	<0.20
Fe	0.13 (7)	<0.25	
Co	0.01 (2)	-	
Cr	0.01 (2)	<0.37	
Mn	0.02 (3)	<0.08	
Mo	0.10 (2)	<0.04	
Ni	0.05 (2)	-	
Zn	0.12 (2)	<0.25	

* Based on a molecular weight of 5.5×10^5 daltons for Ru-P_2 carboxylase (17).

** The metal content of 140- to 300-mg samples of cryst enzyme determined by atomic absorption spectroscopy. The numbers in parentheses indicate the number of carboxylase preparations analyzed for the given metal.

δ The average metal content of 3- to 5-mg samples of two cryst carboxylase preparations determined by emission spectroscopy.

$\delta\delta$ The average copper content of 15- to 25-mg samples of four cryst carboxylase preparations determined by neutron activation. We are most grateful to Dr. W. H. Taylor of this Department for arranging for these analyses at the Phoenix Memorial Laboratory, The University of Michigan.

sufficiently high concentration to be considered as a tightly bound, stoichiometric component of the cryst enzyme. With the notable exception of copper, the results of this metal survey are in excellent agreement with a similar metal analysis of the purified, non-cryst enzyme from spinach (14).

As noted in the introductory remarks, two conflicting reports appear in the literature regarding the content of tightly bound copper in the non-cryst spinach enzyme. Wishnick et al. (14,15), based on analyses by atomic absorption spectroscopy, neutron activation, and electron paramagnetic resonance, reported the presence of 1 g-atom of tightly bound copper (II) per mole of carboxylase. In contrast, Lorimer et al. (4) observed only 0.13 g-atoms of copper per mole of enzyme by atomic absorption analysis. Due to these conflicting reports, the copper content of cryst tobacco Ru-P_2 carboxylase-oxygenase was also determined by neutron activation analysis. The results from this technique were in excellent agreement with those obtained by atomic absorption and emission spectroscopy (Table III), all three analyses indicating that copper was not present in sufficient amounts to be considered as a tightly bound, stoichiometric component of the cryst enzyme.

In summary, the findings of the present study indicate that crystalline Ru-P_2 carboxylase-oxygenase isolated from tobacco does not contain firmly bound copper, iron, or flavin nucleotide, unlike many other crystalline oxygenases examined to date (12, 13). Thus, the enzymatic oxygenation of Ru-P_2 to yield P-glycolate, which is believed to comprise the initial reaction in the photorespiratory metabolism of higher plants (11), appears not to involve these cofactors in the catalytic mechanism.

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