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STRUCTURAL STUDIES OF RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE

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INTRODUCTION

From studies of three crystal forms of ribulose bisphosphate (RuBP) carboxylase from Nicotiana tabacum (called I, II, and III), we have determined the subunit organization of RuBP carboxylase in increasing detail. Combined x-ray diffraction and electron microscope data from these crystals show that there must be some multiple of eight polypeptide chains in the molecule and that the polypeptides are arranged around a fourfold axis of symmetry. At low resolution the eight copies of each polypeptide are equivalent. In more formal terms, the RuBP carboxylase molecule is characterized by point group symmetry D_4 (422, see Figure 1 below). The molecule has a square cross section, about 11 nm on an edge, and a cylindrical channel about 2 nm in diameter which runs along the fourfold axis perpendicular to the square cross section. Four large subunits are arranged in a ring perpendicular to the fourfold axis, and two such rings are eclipsed, forming a two-level structure that extends about 10 nm along the fourfold axis.

Three earlier models for the subunit organization of RuBP carboxylase (summarized in ref. 2) were based on relatively incomplete crystallographic or electron microscope results and on older subunit stoichiometries. All three early models are incompatible with newer, more complete data.

The packing of molecules within crystal form III is similar to the structure of crystallites, believed to be composed of RuBP carboxylase, found in chloroplasts. This suggests that form III may be an in vivo crystal as well as an in vitro crystal form. Our in vitro crystals appear to be suitable for x-ray diffraction studies at least to a resolution of 5 Å, and diffraction data extend to 2.7 Å.

SUBUNIT ORGANIZATION OF RuBP CARBOXYLASE

Conditions for growing form I crystals as large as 1 mm on an edge were developed by Kawashima and Wildman (8). Because of the extreme fragility of the crystals (which collapse to form a drop of water over a film of protein upon the slightest provocation), crystallizing conditions had to be modified to produce the crystals directly within x-ray capillaries (2). X-ray diffraction patterns could then be recorded, but extended only to 50-Å resolution because of the unusually great solvent content of the crystals (79% by weight) and the consequent weak bonding of adjacent molecules. The diffraction pattern was sufficient, however, to narrow the space group to two possibilities, I432 and $I4_132$. Electron micrographs of microcrystalline fragments and of sectioned crystals were found to be compatible with space group $I4_132$. A careful measurement of the density (9), when combined with estimates of the molecular weight, led to the conclusion that there are only 12 molecules in the very large unit cell (Table 1). The minimum molecular symmetry of D_2 (222) follows from these observations (2), since with 12 molecules per unit cell in space group $I4_132$, each must be located on a special position of symmetry D_2 (222).

The 222 symmetry restricts the possible subunit stoichiometries of the RuBP carboxylase molecule. Each subunit must be present in a multiple of four copies. This can be expressed as $L_{4n}S_{4m}$, where L represents the large subunit, S represents the small subunit, and n and m are integers.

Further information on subunit structure was gained from form II crystals (1). X-ray diffraction patterns to a resolution of 15 Å and measurements of crystal density led to the conclusion that RuBP carboxylase molecules must contain at least one fourfold axis, as well as two twofold axes. The method of analysis was similar to that with the form I crystals: x-ray diffraction patterns established that the space group is $P42_12$, and density measurements showed that there are most likely 6 molecules per unit cell. This is possible only if molecules have a fourfold axis of symmetry.

The fourfold molecular axis required by form II is compatible with the three twofold axes demanded by form I if the actual molecular symmetry is D_4 (422). This symmetry requires a multiple of eight copies of each type of subunit, $L_{8n}S_{8m}$. Only a structure of the type L_8S_8 is compatible with the generally accepted molecular weights of the subunits and of the entire molecule. A highly schematic model of RuBP carboxylase is shown in Figure 1, having symmetry D_4 (422) and stoichiometry L_8S_8.

This model is based in part on an electron micrograph (Figure 2a) of a platelet of a form II crystal. This view down the fourfold molecular axis reveals that the molecules are square with a cross section of about 11 x 11 nm. The subunits are arranged about a cylindrical hole about 2 nm in diameter. X-ray diffraction patterns reveal that the square molecule has two layers of subunits along the fourfold axis. Our interpretation of the molecular images in Figure 2 is that one layer of the molecule is formed from four

Table 1

Crystals of RuBP Carboxylase From Tobacco

	Form I	Form II	Form III
Crystallizing: pH	7.4 to 8.8	6.0 to 6.2*	5.2
salts	25 mM Tris	Tris or phosphate	200 mM phosphate 300 mM $(NH_4)_2SO_4$
Crystal morphology	Rhombic dodecahedrons	Square plates & triangular prisms	Pseudo-rhombic dodecahedrons
Density, g cm^{-3}	1.058±0.005	1.096±0.006	1.184±0.007
Space Group	$I4_132$	$P42_12$	$P4_22_12$ or $P4_222$
Dimensions, Å: a=b	383±3	230±2	148.7±0.2
c	383±3	315±3	137.5±0.2
Molecules per cell	12	6	2
Minimum molecular symmetry	D_2	C_2, C_4	D_2 (>5 Å) D_4 (<5 Å)
Finest x-ray resolution, Å	50	14	2.7
Reference	(2)	(1)	(3)

*Form II crystals were subsequently grown at pH 7.8 (Tris buffer) with 70 mM NaCl and 4% polyethylene glycol (MW = 6000). The diffraction pattern of the hkO zone was identical to that from the crystals grown at pH 6.

elliptical units bonded in a square planar ring. These are almost certainly the large subunits. We have no information on the location of the small subunits; their hypothetical placement in Figure 1 is intended only to represent the observed mass distribution of the molecules with a model built from spherical subunits.

The molecular symmetry D_4 (422) is confirmed at low resolution by the form III crystals, which were discovered (3) shortly after form II. To a very good approximation the form III crystals are characterized to about 5-Å resolution by space group I422, with two molecules per unit cell. This requires that each molecule have D_4 symmetry. When crystals are examined at higher resolution, it is found that the site symmetry of molecules is lower, with molecules required only to have D_2 (222) symmetry. This lower symmetry at higher resolution could conceivably reflect the minor difference in amino acid sequences of two types of small subunits, S and S' (7,14). This would require, however, an unusual pattern of assembly, in which S and S' are distributed to form molecules of symmetry D_2. Any other factor causing LS protomers to pair would also cause a reduction in symmetry to D_2.

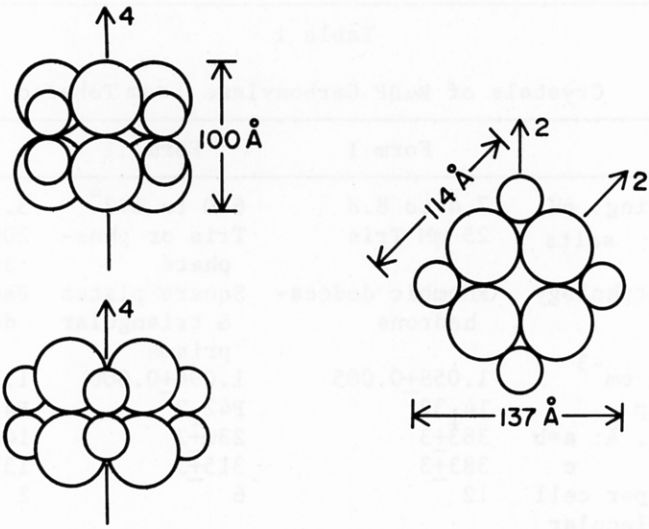

Figure 1. A schematic model of the RuBP carboxylase from tobacco, based on x-ray diffraction and electron microscope studies. The eight large and eight small polypeptide chains are arranged with symmetry D_4 (422). A channel about 20 Å in diameter runs along the fourfold axis, perpendicular to two eclipsed rings of four large subunits. The two rings of four are related by twofold axes. The positions of the small subunits are unknown, and those shown were chosen only to give a molecule with about the same mass distribution as that observed in micrographs, with no compelling reason to suppose that they are accurate.

RELIABILITY OF THE MODEL

To facilitate assessment of the reliability of our model, we should emphasize the assumptions on which it is based. The conclusions on molecular symmetry follow rigorously from the determination of the space group and from the number of molecules in the unit cell. Determination of the space group is unambiguous for form II, and for form III at low resolution. For form I, two space groups are compatible with x-ray diffraction data, but only one is compatible with the electron micrographs of sectioned crystals and of microcrystals.

Correct determination of the number of molecules per unit cell, as explained previously (1, 2), depends on several measurements. The greatest errors are introduced by uncertainties in the molecular weights and the partial specific volumes of protein and liquid of crystallization. In forms I and II, the joint uncertainties in these measurements produce a probable error that barely rules out

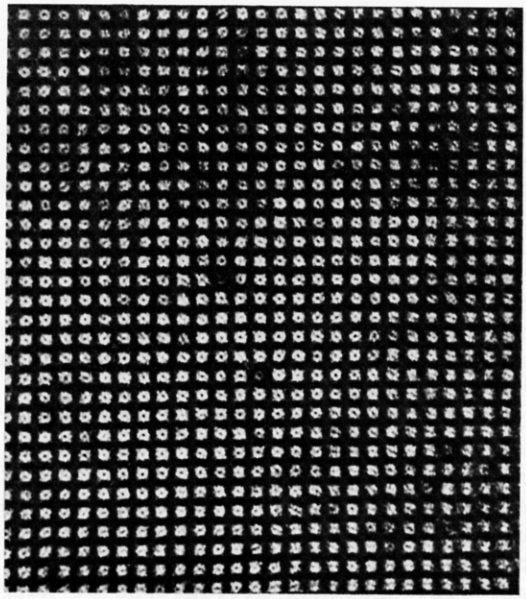

Figure 2. An electron micrograph of a thin platelet crystal of form II. White squares with holes are molecules, viewed down their fourfold axes, and are separated by dark strips of negative stain; nearest neighbors visible are separated by 16 nm.

other conceivable values. Because of the tighter packing in form III, the uncertainty about the number of molecules per unit cell is much less, and the conclusion of D_4 (422) symmetry at low resolution could be wrong only if the molecular weight were greatly in error.

Other conclusions (1) on subunit organization come mainly from form II. The idea of a two-layered structure is based on the measured unit cell size and the micrograph of Figure 2, though the intensity distribution of the diffraction pattern of form II is also consistent with two layers. The suggestion that four elliptical large subunits are bonded in a ring is derived almost entirely from Figure 2. We have no information whatsoever on the positions of small subunits, and the model in Figure 1 is only one of several possibilities consistent with the molecular symmetry and the mass distribution observed in micrographs.

ELECTRON MICROSCOPY OF DRIED, NEGATIVELY-STAINED SOLUTIONS

Molecular images of RuBP carboxylase from several sources have been examined in electron micrographs of dried, negatively-stained solutions. A limitation of this method is that, as the solutions

are dried, the heavy atom stain offers little support, and surface forces between molecules and substrate tend to distort the molecules. Also, damage from the electron beam is severe and tends further to degrade native molecular features.

Baker et al. (2) studied RuBP carboxylase from tobacco by this method and observed poorly defined molecular images, often circular with diameter about 11 nm. Numerous molecules, however, showed a central, stain-filled region about 2 nm in diameter, and several molecules showed a stain-filled line across the molecule. This led Baker et al. to suggest that the RuBP carboxylase molecule contains a unique channel passing through the molecule, which they identified with the fourfold molecular axis. The channel along the axis projects either as a circle or a line, depending on the orientation of the molecule on the substrate.

McFadden et al. (11) studied RuBP carboxylase from Euglena gracilis, and found that it consists of two types of subunits, with molecular and subunit masses similar to those of the tobacco enzyme. Molecular images of the enzyme in micrographs include projections of cubes, and also rosettes with what the authors describe as eightfold symmetry.

Purohit et al. (12) examined micrographs of RuBP carboxylase from Thiobacillus intermedius. This molecule consists of eight copies of a single type of subunit with a molecular weight of about 55,000. The molecular images are circular, many having a central stain-filled hole. Some images are consistent with the eight subunits being arranged in two eclipsed rings of four.

A detailed model was put forward by Bowien et al. (4) for the subunit organization of RuBP carboxylase from Alcaligenes eutrophus. This organism is a facultative, chemolithotrophic, hydrogen bacterium. Its RuBP carboxylase contains eight subunits of molecular weight about 52,000 and eight of molecular weight about 13,000. Molecular images stained with uranyl acetate appear relatively well preserved. Many images display a stain-filled central region, and some molecules seem to have an elongated or double stain-filled region. The molecular diameter is about 13 nm, and the periphery often displays V-shaped projections. Other views of molecules seem to contain three, and in some cases, four parallel layers. Urea-treated samples showed some smaller rings, of diameter 8.5 nm, and also some prominent U- or V-shaped structures.

Bowien et al. (4) suggest that the molecule is characterized by 422 symmetry and is organized in four layers perpendicular to the fourfold axis. Their model is based on two central layers, each made up of four U- or V-shaped larged subunits. Each of the two outer layers is composed of a ring of four spherical small subunits. The rings can be separated from the central layers by urea to produce the smaller ring structures.

The model of Bowien et al. is nearly the same as that of Figure 1 for the positions of the large subunits, which together make up 75% of the mass of the molecule. In fact, the model of Figure 1 is not based on any observations inconsistent with the Bowien four-layer

suggestion. However, the micrograph of Figure 2, and filtered images prepared from it, contain no hint of V- or U-shaped large subunits (2).

In assessing the positions of the small subunits, the cross-linking data of Roy et al. (13) are of interest. These investigators cross-linked the small subunits of RuBP carboxylase from pea leaves with four reagents. All four reagents caused formation of dimers of the small subunit. In addition, small amounts of trimers and tetramers of small subunits were detected with one reagent. These data suggest that small subunits are closely paired in the RuBP carboxylase molecule, and that three or four may be close together. Both the models of Figure 1 and of Bowien et al. are consistent with cross-linked dimers, and the latter is consistent with cross-linked tetramers.

ARE CHLOROPLAST CRYSTALLITES FORM III OF RuBP CARBOXYLASE?

Crystalline inclusions have been observed in chloroplast stroma by electron microscopy (5, 6, 10, 15-17) in both sectioned and freeze-etched chloroplasts, and in both tobacco and spinach chloroplasts. In some cases the crystallites may be induced by the chemical treatments preceding microscopy, but in other cases the crystals are believed to exist *in situ*. Lattice plane spacings from about 6 to 12 nm have been observed in the crystallites, and several investigators have suggested that such spacings are consistent with the crystals being composed of RuBP carboxylase.

We may ask whether any of the *in vitro* crystal forms I, II, or III have lattice spacings compatible with those observed in the inclusion crystallites. Comparison is not entirely straightforward because of several effects. Preparation of specimens for electron microscopy involves steps that can affect lattice dimensions, such as fixation, dehydration, infiltration of polymers, sectioning, staining, and beam damage. Any of these might distort crystallite dimensions. Also, in studies of sectioned crystallites, since chloroplasts are usually sectioned at random, there is no reason why the micrographs could present views along rational crystal directions. In freeze-etch experiments, the replica of the cleaved surface may not lie normal to the direction of view, and this would introduce a distortion in dimensions.

Even with these uncertainties, it is immediately evident that the lattice spacings of forms I and II are too great to match those of chloroplast inclusions. The spacings for form III, however, are consistent with many spacings observed in chloroplast inclusions. A detailed comparison with form III is given in Table 2 for two electron microscope studies of inclusions, and the probable packing of molecules in form III crystals is shown in Figure 3.

In making such comparisons, one must bear in mind that studies of stained sections reflect different dimensions than do studies of

Table 2

Comparison of Lattice Parameters of Crystal Form III With Two Electron Microscope Studies of Chloroplast Inclusion Crystallites

A: Study of Larsson et al. (10) with spinach;
B: study of Willison and Davey (16) with tobacco.

Method	Inclusion parameters	Form III parameters
A. Negatively stained, sectioned inclusions	Parallel lines spaced at 7.5-8.5 nm	d_{111} = 8.4 nm
	Parallel lines spaced at 9.8-11.7 nm	d_{110} = 10.5 nm*
	Two sets of parallel lines with spacings at 8.9-11.0 nm crossing at an angle of about 90°	d_{110} = 10.5 nm* $d_{1\bar{1}0}$ = 10.5 nm Intersection angle = 90°
	Three sets of parallel lines with spacings at 8.5-9.5 nm forming a hexagonal pattern (angles 54-66°)	View along 111 direction d_{101} = 10.1 nm d_{110} = 10.5 nm Intersection angle 59°
B. Negatively stained, sectioned inclusions	Two sets of parallel lines spaced at about 10 nm crossing at an angle of about 90°	View along 001 direction d_{110} = 10.5 nm* $d_{1\bar{1}0}$ = 10.5 nm
Freeze-etched inclusions	Square arrays of molecules with spacings of about 12 nm	View along 001: square* array with spacings 14.9 x 14.9 nm
	Hexagonal arrays with spacings of about 10.4 nm	View along 110: quasi-hexagonal array with spacings of 12.6 and 13.8 nm

*See Figure 3 for an illustration of these spacings.

shadowed replicas of freeze-etched samples. Transmission micrographs of stained sections are projections. Thus, for a centered tetragonal lattice such as form III, the projection along the 001 direction shows the primitive cell dimension $a/\sqrt{2}$, whereas a

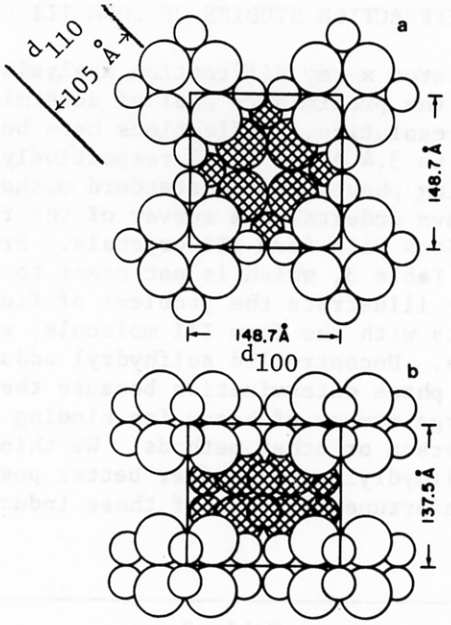

Figure 3. The probable packing of RuBP carboxylase molecules in form III, with the shaded molecule one-half unit cell below the unshaded molecules. (a) View along 001 direction. Note that the intermolecular spacing of 149 Å would be shown by the freeze-etch method, which reveals spacings between molecules in a single plane of the structure. In contrast, an intermolecular spacing of 105 Å would be indicated by transmission electron microscopy, which projects the structure and reveals spacings between molecules regardless of depth into the crystal. (b) View along 100 direction.

shadowed replica of the crystal surface shows the centered cell dimension a. This is why, in Table 2, the view along the 001 direction yields different dimensions for these two types of observations.

Evidence against the chloroplast inclusion crystallites being form III is that form III crystals have so far been grown only at pH around 5.2. When transferred to solutions at pH 7, they dissolve unless sulfate or phosphate is present at high concentration. In chloroplasts the pH is well above the range of stability of form III crystals, but there may be compensating factors. Dr. Fenna in our laboratory has recently found conditions (footnote to Table 1) under which form II is stable at a pH value 1.6 units above the original crystallizing conditions if polyethylene glycol is present.

In summary, the lattice spacings of form III are consistent with many observations on chloroplast inclusions, but identity of form III with chloroplast structures has not been definitely established.

X-RAY DIFFRACTION STUDIES OF FORM III CRYSTALS

We have initiated x-ray diffraction analysis of the structure of form III, with the preliminary goal of determining the molecular structure to 5-Å resolution. Reflections have been recorded in the hkO and hOℓ zones to 3.4 Å and 3.7 Å respectively.

For determining phases by the standard method of isomorphous replacement, we have undertaken a survey of the reactions of various heavy metal ions with form III crystals. Preliminary results are summarized in Table 3, which is not meant to be comprehensive but is intended to illustrate the problems of finding suitable heavy metal adducts with the form III molecule, which contains 96 cysteinyl residues. Uncontrolled sulfhydryl adducts are probably not promising for phase determination because the chances are small for locating a large number of heavy ion binding sites within the crystal with Patterson or other methods. We think heavy ions that do not attract sulfhydryl groups offer better possibilities of usefulness, but unfortunately some of these induce disorder in the crystals.

Table 3

Effects of Heavy Metal Ions on Form III RuBP Carboxylase Crystals

Compound	Concentration of heavy metal relative to LS pair	Crystal disorder	Good diffraction pattern with intensity changes
CH_3HgCl	1	No	Yes
o-Chloromercuri-phenol	1	Yes	---
PCMBS	1	Yes	---
Mercury-cyclo-hexane-butyrate	5-10	Yes	---
Mercurochrome	5-40	Yes	---
Baker mercurial	2-4	Yes	---
Uranyl nitrate	1-2	No	Yes
	>2	Yes	---
$PtCl_4$	5-10	Yes	---
K_2PtCl_6	5	Yes	---
$K_2Pt(CN)_4$	400-1000	No	Yes
	>1500	Yes	---
$KAu(CN)_2$	200-1500	Yes	---
Thallium acetate	5-30	No	Yes
	>40	Yes	---
Nb_6Cl_{14}	20	No	No (disordering along c*)

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DISCUSSION

PAECH: Do you know whether the crystalline enzyme is able to catalyze the carboxylase reaction?

EISENBERG: No. We have not looked into that.

WILDMAN: The remarkable property of the crystals is that, the instant they are put into RuBP, they dissolve. They are stable in solutions containing CO_2 and Mg^{2+}, however. The crystal dissolved in RuBP can be dialyzed for a prolonged period without recrystallizing, but upon addition of Mg^{2+} and CO_2 it crystallizes immediately.