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Crystallisation and preliminary X-ray data of ribulose-1,5-bisphosphate carboxylase from spinach

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Crystals of a tertiary complex of spinach ribulose-1,5-bisphosphate carboxylase/oxygenase with the activators Mg^{2+} and CO_2 have been grown. These crystals diffract strongly to 1.6 Å resolution. The spacegroup is $C222_1$ with unit cell dimensions a = 158.6 Å, b = 158.6 Å, c = 203.4 Å. Additional local symmetry is apparent in the pattern of absences and the intensity distribution of the X-ray precession photographs. The photographs have been interpreted in terms of a molecule (consisting of eight large and eight small subunits, L_8S_8) with 222 symmetry and a molecular centre shifted 2 Å in the x direction from the origin of the unit cell. The asymmetric unit contains half the L_8S_8 molecule. The intensity distribution suggests that the molecular symmetry does not deviate far from 422. These crystals are compared with other crystalline forms of the enzyme and the implications of these results are discussed.

Key words: carboxylase/crystallisation/purification/ribulose-bis-phosphate/X-ray

Introduction

The enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (3-phospho-D-glycerate carboxy-lyase (dimerising), EC 4.1.1.39), is present in large quantities in plant leaves and has a crucial role in carbon dioxide fixation in chloroplasts. Under normal atmospheric conditions, it is bifunctional, directing the flow of carbon to either the photosynthetic carbon reduction cycle or to the photo-respiratory cycle (Lorimer, 1981). Activation of the enzyme by formation of the complex enzyme-Mg^{2+}-CO_2 is required for both carboxylase and oxygenase activities. Activation is reversible and is dependent on pH as well as concentrations of carbon dioxide and magnesium ion. The activator carbon dioxide molecule is distinct from the substrate carbon dioxide for the carboxylation (Lorimer *et al.*, 1976).

The spinach enzyme, in common with the enzyme from other higher plants, is oligomeric (mol. wt. 560 kd), containing eight large (55 kd) and eight small (15 kD) subunits. There appears to be considerable sequence homology between the large (catalytic) subunits of different species but the amino acid compositions of the small subunits are very variable.

Crystals suitable for X-ray analysis have been grown from the tobacco enzyme (Baker *et al.*, 1977b) and a similar crystalline form has been reported for the potato enzyme (Johal *et al.*, 1980). However, for neither species have crystals been grown under conditions corresponding to activation. Crystals have been grown in the presence of carbon dioxide and magnesium ion for the enzyme from the bacterium *Alcaligenes eutrophus* (Bowien *et al.*, 1980), and very recently crystals of a quaternary complex of the spinach enzyme have been grown (Andersson and Brändén, 1983).

This paper reports a modified purification of spinach Rubisco and the growth and characterisation of crystals grown under carbon dioxide and magnesium ion concentrations and at a pH corresponding to maximum activation. Diffraction extends to higher resolution than previously reported for this enzyme.

Results

The enzyme Rubisco has been extracted from spinach leaves by a method modified from those previously described (Baker *et al.*, 1977a; Poulsen and Lane, 1966; Chan *et al.*, 1972) and detailed in Materials and methods. Crystals have been grown under conditions similar to those in which the enzyme shows maximum activity in an effort to obtain crystals of the enzyme in the activated state. Crystals of three different morphologies have been observed under the conditions given in Table I. Crystals of type P appear to be derived from tetragonal bipyramids and grow to < 100 μm in diameter. We have not been able to obtain X-ray diffraction photographs of them. Type R crystals are flakes with two dimensions of order 0.5 mm and the third too thin to measure. They are too thin for study by X-ray diffraction. Under comparable conditions, material suitable for electron microscopy has been obtained.

Type Q crystals are flat plates, typically 1.2 x 0.8 x 0.15 mm in dimension. X-ray diffraction photographs have been taken of these crystals and are shown in Figure 1. Figure 1a was obtained using the synchrotron source at L.U.R.E., Orsay. The beam was parallel to the shorter plate edge. Figures 1b, 1c, 1d show 12° precession photographs about three orthogonal principal axes giving **hk0**, **0kl** and **h0l** zones and were taken on a conventional X-ray source.

The precession photographs show **mm** symmetry and general absences such that for a reflection to be present **h** + **k**

Table I. Components of crystallisation media for growth of crystals from spinach Rubisco

Component	Crystal type		
	P	*Q*	*R*
Rubisco concentration (mg/ml)	2.5–5.0	2.5–8.0	1.0–2.5
PEG 8000 (% w/v)	6–10	7.2	6–8
$MgCl_2$ (mM)	0–5	10	20–50
$NaHCO_3$(mM)	0–20	15–25	25
pH	6.8–8.0	7.6–8.0	8.2–8.6
NaCl (mM)	15–100	15–40	15–40
Temperature (°C)	4–20	20	20, 30

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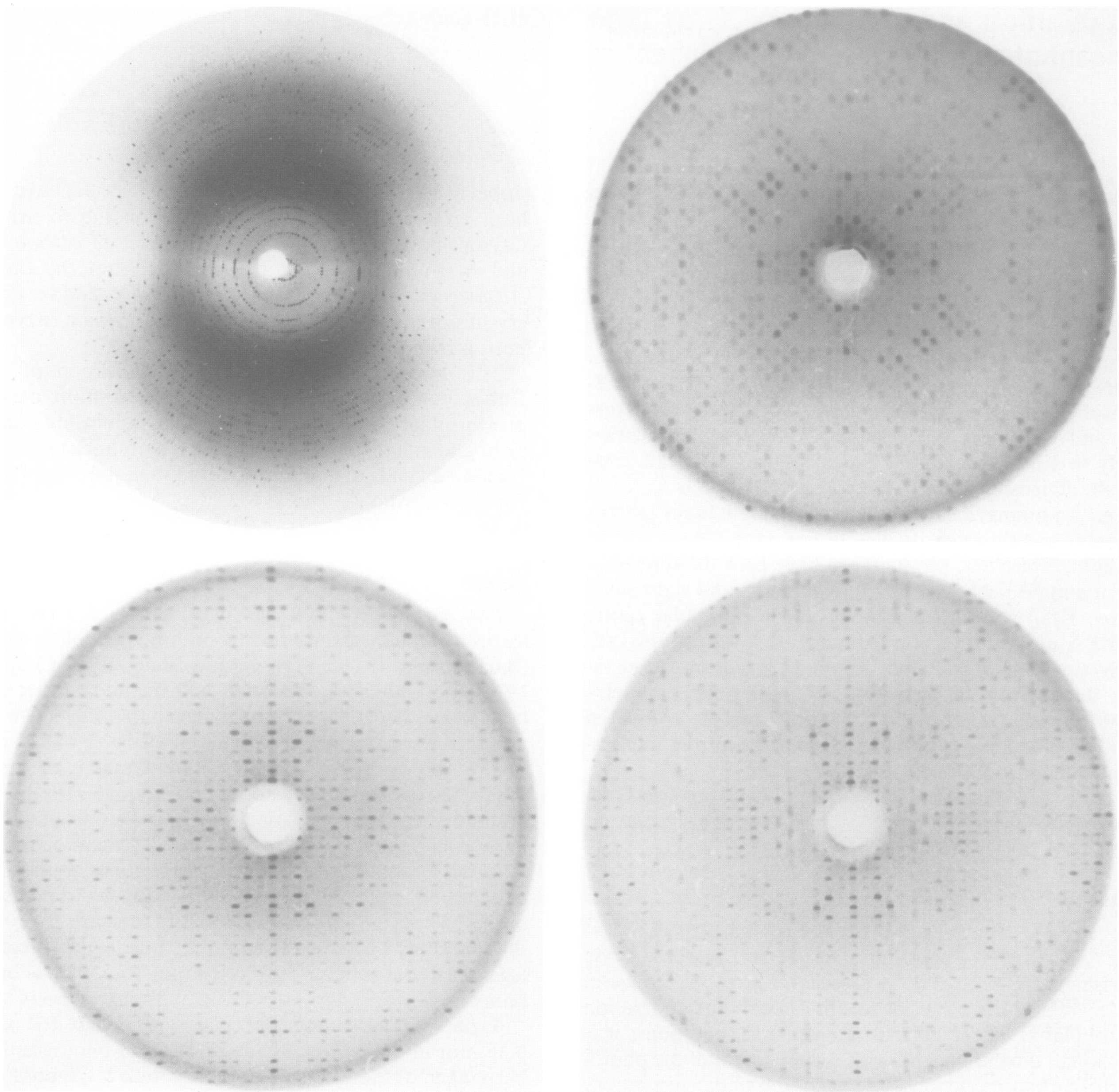

Fig. 1. X-ray diffraction photographs of Rubisco **(a)** still; **(b)** 12° precession photograph of **hk**0 zone; **(c)** 12° precession photograph of 0**kl** zone; **(d)** 12° precession photograph of **h**0**l** zone.

Table IIa. Additional absences: index relationship required for reflection to be present on **h**0**l** zone

Range of **h** (**h** = 2*n*)	Restriction on **l**
0– 4	$\lvert \mathbf{l} \rvert = 2n$
6–14	$\lvert \mathbf{l} \rvert = n$ (no restriction)
16–24	$\lvert \mathbf{l} \rvert = 2n + 1$
26–32	$\lvert \mathbf{l} \rvert = n$ (no restriction)
34–42	$\lvert \mathbf{l} \rvert = 2n$

Table IIb. Intensity restrictions on **hk**0 zone: index relationship required for reflection to be present unless very weak

Range of **h**	Range of **k**	Index relationship
≤11	$11 < \lvert \mathbf{k} \rvert < 30$	$\mathbf{h} = 2n + 1$, $\mathbf{k} = 2n + 1$
all	$0 \le \lvert \mathbf{k} \rvert \le 6$	$\mathbf{h} = 2n$, $\mathbf{k} + 2n$
	$\lvert \mathbf{k} \rvert > 30$	

= 2*n*. Further, for the 0**kl** zone **k** = 2*n* and **l** = 2*n*. The absences are consistent with the spacegroup $C222_1$ with four molecules present in special positions of the type *x*,0,0. The cell dimensions are a = 158.6 ± 0.5 Å, b = 158.6 ± 0.5 Å, c = 203.4 ± 0.7 Å, ($\alpha = \beta = \gamma = 90°$). The cell volume is consistent with four molecules of Rubisco (L_8S_8, mol. wt. 560 kd) per unit cell.

There is evidence of additional local symmetry. The **h0l** zone shows the additional absences shown in Table IIa. The **hk0** zone shows a further pattern of absent or very weak reflections given in Table IIb. Additionally there is an approximate 422 symmetry of the intensity distribution over this zone although there is certainly no 4-fold crystallographic symmetry.

Discussion

Crystals of Rubisco have been grown from several plant

Table III. Crystal forms of oligomeric L_8S_8 Rubisco from various species

Species	Crystallising medium	pH	Other metabolites	Crystal type	Spacegroup	Higher symmetry spacegroup	Extent of diffraction	Molecules/ asymmetric unit	Required molecular symmetry	Reference
Nicotiana tabacum	0.025 M Tris/HCl	7.4–8.8	–	I	$I4_132$	–	50 Å	1/4	222	a
Nicotiana tabacum	0.025–0.050 M Tris/HCl or 0.025 M K phosphate	6.0–6.2	–	II	$P42_12$	–	14 Å	1/4 and 1/2	4 and 2	b
Nicotiana tabacum	0.2 M K phosphate 0.2–0.3 M $(NH_4)_2SO_4$	4.8–5.2	–	III	$P4_22_12$	I422	2.7 Å	1/4	222	c
Nicotiana sylvestris	0.2 M K phosphate 0.01 M NaCl vapour diffusion against	5.4	–	III	$P4_22_12$	I422	n.r.	1/4	222	d
Potato	0.025 M K phosphate 0.1875 M $(NH_4)_2SO_4$	4.8–6.2	–	III	$P4_22_12$	I422	n.r.	1/4	222	d
Alcaligenes eutrophus	0.02 M Tris/HCl dialysis against 25% (sat) Na_2SO_4	7.8	10 mM $MgCl_2$ 50 mM $NaHCO_3$	–	$P4_22_12$	–	3.5 Å	1/4	222	e
Spinach	0.01–0.02 M Tris/NaCl 0.05 M K phosphate PEG 6000 4–10% w/v	6.7	CABP	B	$P2_12_12$	–	n.r.	1/2	2	f
	dialysis against PEG 6000	6.7	CABP 2.5 mM $MgCl_2$	C	I422	–	3.0 Å	1/2	2	f
Spinach	0.025 M K phosphate 0.84 M $(NH_4)_2SO_4$ vapour diffusion against $(NH_4)_2SO_4$	7.0–7.3	CABP 2.5 mM $MgCl_2$ 5 mM $NaHCO_3$	D	C222	F422	2.0 Å	1/2	2	g
Spinach	0.038 M bicine/NaOH PEG 8000 7.2% w/v	7.8	25 mM $MgCl_2$ 10 mM $NaHCO_3$	Q	$C222_1$	P422	1.6 Å	1/2	2	h

[a]Baker *et al.*, 1975; [b]Baker *et al.*, 1977a; [c]Baker *et al.*, 1977b; [d]Johal *et al.*, 1980; [e]Bowien *et al.*, 1980; [f]Andersson *et al.*, 1983; [g]Andersson and Brändén, 1983; [h]This paper.
n.r. not reported.

and bacterial species under varying conditions. Those for which X-ray measurements have been made are summarised in Table III. Some of these studies have been combined with electron microscopy and have suggested that the Rubisco molecule may have 422 symmetry (Baker *et al.*, 1977a, 1977b). In the crystal form of the enzyme discussed here, if the molecular symmetry does not deviate far from 422, the apparent 4-foldness of the intensity distribution of the **hk0** zone suggests the 4-fold axis should be approximately parallel to, though not coincident with, the crystallographic *c* axis. One molecular 2-fold axis is required to be co-incident with the *a* axis and the final 2-fold axis must thus be approximately parallel to *b*.

The pattern of the additional absences on the **h0l** zone can be explained in terms of molecules with 222 symmetry in the spacegroup $C222_1$. The molecules centred at x,0,0 and $\bar{x}$, 0, ½ are $2x$,0,½ apart and the phase shift between them is $2\pi(\frac{2x\mathbf{h}}{a} + \frac{\mathbf{l}}{2})$. Reflections appear for $\mathbf{l} = 2n$ where $\mathbf{h} = 0$ and again where $|\mathbf{h}| = 34 - 42$; the centre of this region is $|\mathbf{h}| = 39$. This is the first repeat and the phase difference is 2π where the difference in **h** index is 39 and that in **l** index 0.

i.e., $2\pi\,(\frac{2.x.39}{a} + \frac{0}{2}) = 2\pi$

$$\frac{2.x.39}{158.6} = 1$$

thus $x = 2.03$ Å.

The centres of the two L_8S_8 molecules are thus 2.03 Å from the origin of the $C222_1$ cell in the x direction. Figure 2 shows a schematic packing diagram of the molecules in the $C222_1$ cell.

If this distance were zero, with the molecules in the same orientation with 222 symmetry, the local axes would be co-incident with crystallographic 2-fold axes in the spacegroup C222 with the *c* axis halved and ¼ molecule in the asymmetric unit (see Figure 3). Further, if the molecular symmetry were 422, the spacegroup would be P422 with $a = b =$ 113 Å, $c =$ 102 Å and ⅛ of the molecule (i.e., a single LS protomer) in the asymmetric unit. This is illustrated in Figure 4.

The possibility that the crystallographic symmetry is 222 and that the crystals suffer from a stacking fault in *c* has been considered. No simple disordered model has yet been devised which would explain the pattern of absences given in Table II. Similarly, while twinning of crystals cannot be rigorously excluded, no mode of twinning of the higher symmetry cell with a single layer of molecules in the *c* direction has been found which would explain the presence of reflections with odd ℓ value. All these features of the pattern can be explained on the basis of the $C222_1$ cell described already.

It is remarkable that crystals of the spinach enzyme containing the activators and the transition state analogue 2-C-carboxy-D-arabinitol-1,5-bisphosphate (CABP) which have been grown from ammonium sulphate have very similar cell dimensions [$a = b =$ 157.2 Å, $c =$ 201.3 Å] and a closely related spacegroup (C222) Andersson and Brändén (1983). The pattern of additional absences is, however, quite different from that described here and the arrangement of the molecules in the cell is not the same.

In none of the crystals of this enzyme studied so far does 422 crystallographic symmetry exist. In only one form (form II from *Nicotiana tabacum*) is there a crystallographic 4-fold

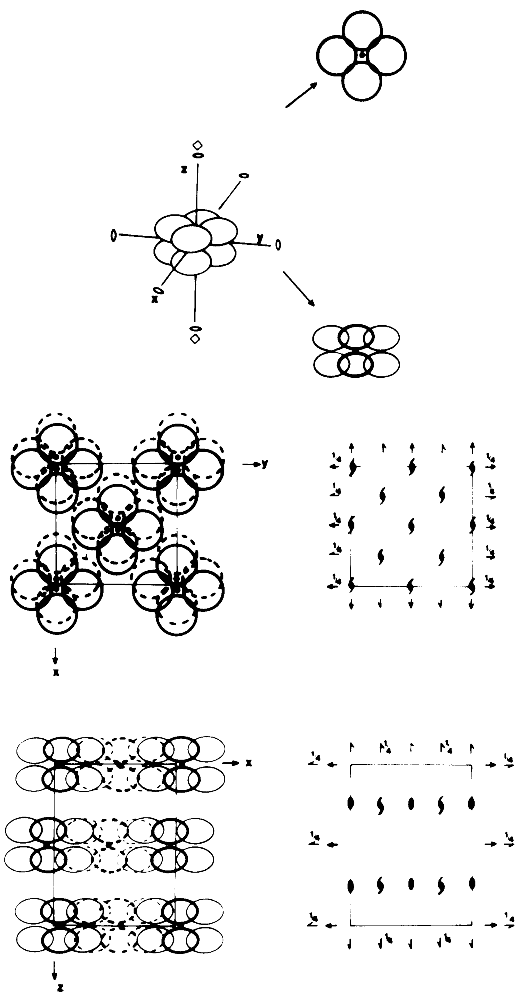

Fig. 2. (a) A schematic representation of the Rubisco oligomer L_8S_8 (one LS unit is represented by an ellipse) and projections down *z* (above) and *y* (below). Bold lines signify two superimposed LS units, light lines a single LS unit. **(b)** The packing of Rubisco molecules in the $C222_1$ cell and the symmetry elements of the spacegroup. (Solid lines represent molecules centred at 0, dashed lines molecules centred at ½; other conventions as in **a**.)

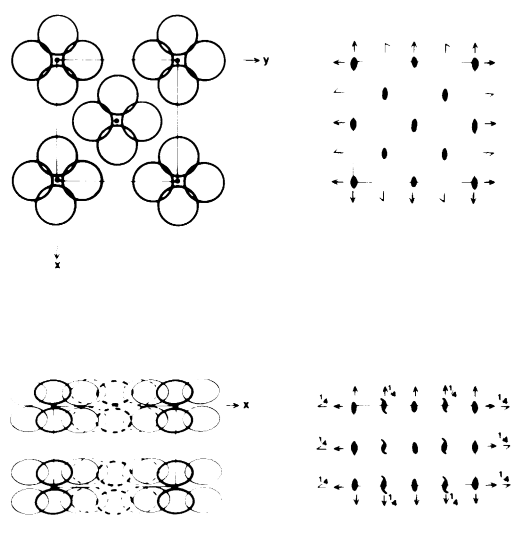

Fig. 3. The C222 cell which arises if the Rubisco molecules are exactly superimposed down the *c* axis. (Conventions as in Figure 2.)

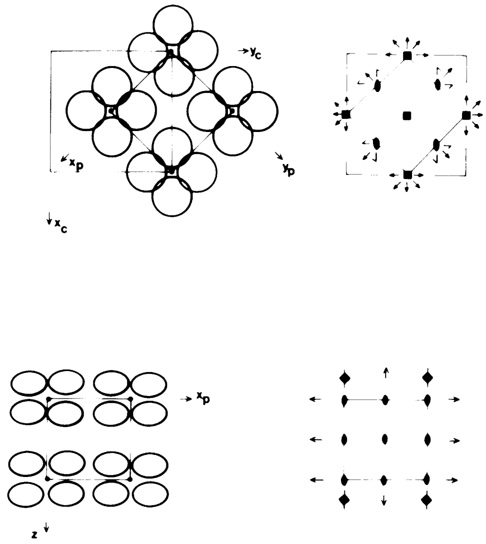

Fig. 4. The P422 cell which arises if the Rubisco L_8S_8 unit has 422 symmetry and the molecular axes are aligned with the cell axes. (Conventions as in Figure 2.).

axis and in this form, the pattern extends to only 14 Å resolution. None the less, in the form III crystals from tobacco there is 422 symmetry at low resolution and in form D from spinach and the form described here some evidence for at least approximate local 422 symmetry. It still remains unclear, therefore, whether the Rubisco molecule has strictly 422 symmetry in either the activated or the unactivated form. The crystals grown, here under activating conditions for the spinach enzyme, require there to be only exact 2-fold symmetry. The crystals of the bacterial *A. eutrophus* enzyme, also grown under activating conditions show 222 crystallographic symmetry, but the identity of each individual protomer has yet to be established for any species.

Materials and methods

Materials

Sephadex G-25 and Sepharose 6B were from Pharmacia Fine Chemicals; polyethylene glycol 8000 and ribulose-1,5-bisphosphate from Sigma. The remaining chemicals were reagent grade. Spinach leaves were from ~6-week-old plants, grown under natural conditions in the Botany School garden.

Purification of the enzyme

The enzyme was isolated and purified by a procedure derived from a combination of those developed in other laboratories (Poulsen and Lane, 1966; Chan *et al.*, 1972; Brown *et al.*, 1980). Fresh spinach leaves are demi-ribbed, washed and wiped with filter paper; 200 g are ground in a mixer at 4°C with 400 ml 'extraction buffer' (50 mM Tris, 1.0 M NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 5 mM dithioerythreitol, adjusted to pH 7.1 at 20°C with HCl) containing 10 g insoluble polyvinyl polypyrrolidone. The paste is passed through cheesecloth and the deep green extract centrifuged at 10 000 *g* for 15 min at 4°C. Solid ammonium sulphate is then added to the supernatant to 30% saturation in the cold. The supernatant is brought to 50% saturation and the precipitate resuspended in ~25 ml of cold extraction buffer and recentrifuged at 130 000 *g* for 2 h at 4°C. The supernatant is passed through a column (20 x 5 cm diameter) of Sephadex G-25, equilibrated in buffer 'B' (25 mM Tris, 0.2 M NaCl, 0.5 mM EDTA, adjusted to pH 7.4 at 20°C with HCl) at room temperature. The fractions containing protein (~35 ml) are made up to 32% saturation with ammonium sulphate at room temperature; the supernatant is brought to 47% saturation and the precipitate resuspended in ~10 ml of cold buffer 'B'. The resulting brownish solution is applied to a column of Sepharose 6B (80 x 2.5 cm in diameter equilibrated in buffer 'B' at 4°C). The fractions containing activity and with an optical density $A_{280\ nm} > 1.6$ are pooled (~60 ml), precipitated with solid ammonium sulphate (to 55%) resuspended in 7–10 ml of buffer 'B' at 4°C and rechromatographed in the same column. Fractions are pooled, concentrated in the same way and resuspended at a concentration of ~40 mg/ml, in 5 ml of buffer 'B' at 4°C. The enzyme is kept in these conditions, under which it is stable for several weeks. The preparation is centrifuged before use. Protein concentration is determined from O.D.$_{280\ nm}$ measurements using $E^{1\%}_{280}$ = 15.0.

The carboxylase activity is measured by following the formation of 3-phosphoglycerate spectrophotometrically at 30°C (Lilley and Walker, 1974; Barcena, in preparation). Oxygenase activity is measured following ribulose-1,5-bisphosphate-dependent uptake of oxygen in an oxygen electrode using 'CO_2 free solutions' (Lorimer *et al.*, 1977; Barcena, in preparation). The enzyme was activated before assays (Lorimer *et al.*, 1977, Barcena, in preparation). The carboxylase activity varied between 0.8 and 1.2 μmol CO_2 fixed/min/mg and the oxygenase between 0.07 and 0.08 μmol O_2 fixed/min/mg.

Crystallisation

Crystals of type Q (Table I), suitable for crystallographic studies were grown as follows: 15 μl of enzyme prepared as above (40 mg/ml) in buffer 'B' were mixed gently with 185 μl of crystallisation medium in an Eppendorf microfuge polypropylene tube and allowed to stand for 7 – 10 days at 20°C. Crystals grew to their final size in 10 – 15 days. The final composition of the crystallising medium was: 30 – 40 mM bicine, 20 – 25 mM $NaHCO_3$, 10 mM $MgCl_2$, 25 mM NaCl, 7.2% (w/v) PEG-8000, 3 mg/ml enzyme (2 mM Tris.HCl, 25 μM EDTA from the enzyme solution). The pH was adjusted to 7.9 with NaOH.

X-ray diffraction measurements

Initial still photographs were taken using the synchrotron source at L.U.R.E. (Orsay). The beam characteristics were: 1.72 GeV. 292 mA, λ = 1.40 Å. 12° precession photographs were taken on a conventional Elliot GX6 rotating anode tube using CuK_{α} radiation λ = 1.5418 Å at 39 kV, 39 mA.

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