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PURIFICATION AND CHARACTERIZATION OF MAIZE RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE

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Abstract— The ribulose-1,5-bisphosphate carboxylase/oxygenase purified from maize (a C_4 monocot) to homogeneity has a MW of 532 000 and sedimentation coefficient ($S_{20,w}$) of 19.1 The purified enzyme has a specific activity of 2.36 μmol CO_2 fixed/min mg protein at 30° Dissociation of the holoenzyme by sodium dodecyl sulfate yielded large subunits of MW 53 500 and small subunits of MW 13 000 Analytical isoelectrofocusing of *S*-carboxymethylated enzyme revealed three major large subunit polypeptides and two major small subunit polypeptides. The partially purified enzyme has an isoelectrofocusing point of pH 4 6 Sulfhydryl group determinations indicated 78 for sodium dodecyl sulfate denatured holoenzyme, eight for the large subunit, and two for the small subunit The activity of ribulose-1,5-bisphosphate carboxylase decreased during sulfhydryl titration indicating that cysteine is involved in catalysis by the enzyme The total amino acid residues in the holoenzyme and separated large and small subunits are 4843, 494 and 112, respectively The amino acid composition of the holoenzyme and subunits are very similar to the data obtained from several C_3 species These results suggest that ribulose-1,5-bisphosphate carboxylase/oxygenase has been highly conserved during evolution.

INTRODUCTION

Ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (EC 4.1 1 39) catalyses the carboxylation [1] of RuBP to form two molecules of 3-phosphoglycerate and the oxygenation [2] of RuBP to form one molecule each of 3-phosphoglycerate and phosphoglycolate Higher plant RuBP carboxylase/oxygenase (RuBPCase) is an oligomer composed of eight large subunits (LSU), MW 52 000–58 000, and eight small subunits (SSU), MW 12 000–18 000 [3] The LSU contains the catalytic sites for both the carboxylase and oxygenase activities [3]. The function of the SSU is not known. The biosynthesis of RuBPCase involves transcription and translation at two different sites in the leaf cell The LSU is encoded by chloroplast DNA and is synthesized in the chloroplast [4, 5] The SSU is encoded by nuclear DNA and is synthesized in the cytoplasm as a large precursor molecule before being transported into the chloroplast and processed into a functional subunit [6, 7] RuBPCase is localized in chloroplasts of all leaf chlorenchymatous cells of C_3 and CAM plants [8]. In C_4 plants RuBPCase is present in chloroplasts of bundle sheath cells only [8–10]. It is absent in the mesophyll cells of C_4 plants [8–10] even though their chloroplast DNA contains the gene for the LSU [11].

Abbreviations PVP, polyvinylpolypyrrolidone, Hepes, (*N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid); Tris, Tris(hydroxymethyl)aminomethane; DTT, DL-dithiothreitol, SDS, sodium dodecyl sulfate, DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid)

Recent comparative studies [12, 13] of kinetic properties of RuBPCase from phylogenetically diverse plants have revealed variation in $K_m(CO_2)$ values associated primarily with the differences in photosynthetic pathway. The enzyme from C_3 and CAM species exhibits lower $K_m(CO_2)$ values than does that from C_4 species The lower affinity for carbon dioxide of the C_4 enzyme is thought to be associated with the carbon dioxide-concentrating mechanisms in C_4 plants. Thus, it is of great interest to compare some important physical and chemical properties of the enzyme from C_3 and C_4 species. There are numerous reports on the isolation and characterization of RuBPCase from C_3 plants [14–19]; however, similar information on the C_4 enzyme is lacking. In this study, we have purified RuBPCase from a classical C_4 plant, *Zea mays*, and characterized some important physicochemical properties of the enzyme

RESULTS

Enzyme purification

Leaf tissue was monitored microscopically during blending to determine breakage of the bundle sheath cells While the blend time used did not give 100% breakage of bundle sheath cells it was deemed sufficient without being detrimental to released RuBPCase. Table 1 shows the results of the enzyme purification. The step gradient was useful in separating considerable PEP carboxylase from RuBPCase even though some RuBPCase was lost. The remaining PEP carboxylase was eliminated by linear sucrose density gradient centrifugation. Fractions having RuBPCase activity only were pooled for further purification Sepharose-6B chromatography separated the re-

Table 1 Purification of RuBPCase from maize leaves

Step	Total protein* (mg)	Specific activity† (units/mg)	Total activity (units)	Yield (%)
1 Crude extract	4335	0 22	954	100
2 35–60% saturated $(NH_4)_2SO_4$ fraction	1145	0 67	767	80
3 35% sucrose in 60% $(NH_4)_2SO_4$ step gradient	361	1 56	563	59
4 0 29–0 84 M linear sucrose density gradient	140	2 34	328	34
5 Sepharose 6-B chromatography	88	2 36	208	22

*Starting material, 250 g fr wt leaf

†Sp act expressed as μmol CO_2 fixed/min mg protein determined at 30

maining nucleic acids from RuBPCase The $A_{280/260}$ ratio was 1 93 following this step indicating the absence of nucleic acids

Purity and physiochemical properties

The purified RuBPCase was completely homogenous according to non-denaturing and SDS slab polyacrylamide gel electrophoresis (which separated the enzyme into LSU and SSU moieties) and to sedimentation velocity ultracentrifugation The subunits of the purified RuBPCase after carboxymethylation were isolated by gel filtration on Sephadex G-100 (Fig. 1) The purity and MW of the subunits were determined by SDS slab gel electrophoresis. On this basis the MW of the holoenzyme was estimated to be 532 000 and the $S_{20,w}$ value determined by sedimentation coefficient was 19 1 MWs of the subunits, determined from SDS dissociation of native RuBPCase and analysis of relative mobilities on SDS slab gel electrophoresis were 53 500 and 13 000 for the LSU and SSU, respectively These MWs are in good agreement with those determined for wheat [19], a C_3 Gramineae species, 53 000 and 13 500 for the LSU and SSU, respectively. Higher plant RuBPCase has MWs of 51 000–58 000 for the LSU and 12 000–18 000 for the SSU [3] Carboxymethylated LSU showed an increased MW, 57 500 compared to 53 500 for non-carboxymethylated. The 14% acrylamide SDS slab gel could not distinguish carboxymethylated from non-carboxymethylated SSU The sedimentation coefficient of maize RuBPCase is close to that determined for the enzyme from C_3 species [15]

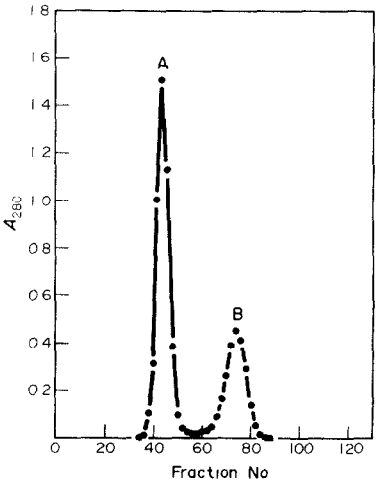

Fig 1 Separation of subunits of RuBPCase by gel filtration on Sephadex G-100 in the presence of urea following *S*-carboxymethylation The large and small subunits are represented by A and B, respectively

Comparison of RuBPCase polypeptide composition from a variety of higher plants, including two genera of C_3 Gramineae species, revealed characteristically three LSU polypeptides and one–four SSU polypeptides [20] In the present study analysis of carboxymethylated RuBPCase from maize, a C_4 species, by 2D-urea-IEF/SDS-PAGE yielded, consistently, three major LSU polypeptides and two major SSU polypeptides Table 2 summarizes the isoelectric points of these polypeptides Maize RuBPCase polypeptides focused at a slightly lower pH than generally observed for other plant species In general, LSU polypeptides focus near pH 6 0 and SSU polypeptides near pH 5 0

Interestingly, 2D-urea-IEF/SDS-PAGE analysis of non-carboxymethylated maize RuBPCase still revealed only two major SSU polypeptides and two, possibly three, major LSU polypeptides According to previous work [21], additional polypeptides might have been revealed due to oxidation of thiol groups.

Isoelectric focusing of a partially purified RuBPCase preparation from maize leaf under non-denaturing conditions resulted in two major protein bands (Fig 2) Based on enzyme activity the protein band which focused at pH 4 6 was identified as RuBPCase while the band which focused at pH 5 0 was PEP carboxylase The maize RuBPCase has a lower apparent p^I (p^I) than those reported for C_3 RuBPCase, although direct comparison of isoelectric focusing results between laboratories is difficult due to variations in duration and temperature of focusing and concentration of carrier ampholyte employed Pea leaf RuBPCase focuses at pH 5 25 [22]. In spinach, RuBPCase and PEP carboxylase migrate to-

Table 2 Isoelectric points of the subunit polypeptides of carboxymethylated maize leaf RuBPCase

Polypeptide	Isoelectric point
L1	5 28
L2	5 24
L3	5 21
S1	4 67
S2	4 51

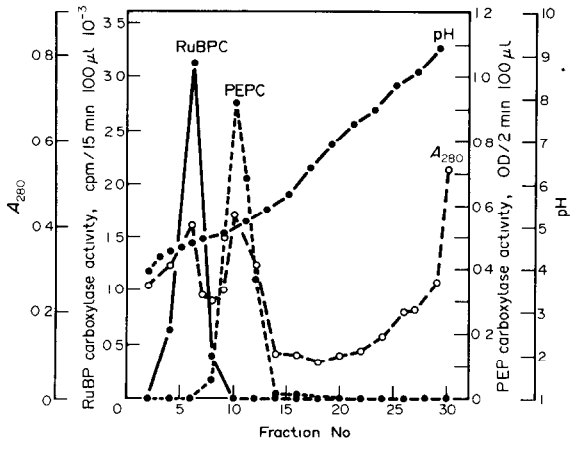

Fig 2 Isoelectric focusing preparative flat-bed gel of a partially purified enzyme sample. Electric focusing was followed by fractionation of the flat-bed gel and determination of enzyme activities, RuBPCase and PEP carboxylase, *A* at 280 nm and pH

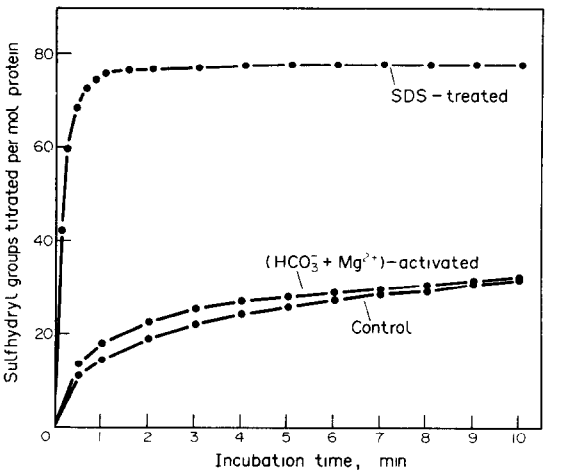

Fig. 3 Titration of free sulfhydryl groups of purified RuBPCase, SDS-treated and fully activated enzyme, with 5,5'-dithiobis-(2-nitrobenzoic acid)

gether electrophoretically, with a $p^{I'}$ of 6.25 [23]. The p^I values of RuBPCase from potatoes [24], cucumber and lettuce [25] ranged from 6.2 to 7.0. These results suggest that RuBPCases from various sources may have very different net charges.

There has been some concern over whether cysteinyl sulfhydryl groups of the RuBPCase are an integral part of the catalytical region [26] The number of free sulfhydryl groups and the effect of modification of them on enzyme activity were determined using a mild alkylating agent, DTNB, the Ellman's reagent Table 3 summarizes the number of free sulfhydryl groups of maize RuBPCase titrated under various conditions. At saturating DTNB concentrations 76 sulfhydryl groups/mol holoenzyme were titrated. With enzyme activated with HCO_3^- and Mg^{2+}, 72 sulfhydryl groups/mol were titrated. Upon denaturation with SDS, 78, 8 and 2 sulfhydryl groups/mol protein were titrated for the holoenzyme and its large and small subunits, respectively. Denaturation of RuBPCase resulted in rapid (2 min) titration of sulfhydryl groups (Fig. 3). In contrast, titration of native RuBPCase, as well as activated RuBPCase, was much slower, of the order of a few hours (Figs 3 and 4); however, the extent of sulfhydryl group titration was about the same as with native RuBPCase (Table 3) For the spinach (C_3) RuBPCase 96 sulfhydryl groups/mol enzyme protein has been reported [27]. Recently, Yeoh *et al.* [19] reported, based on amino

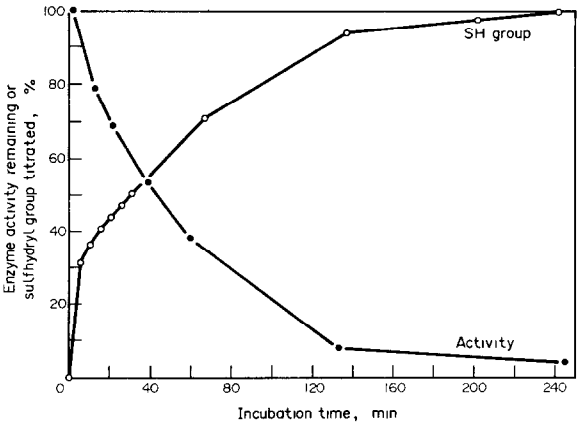

Fig. 4. Residual RuBPCase activity with titration of free sulfhydryl groups with 5,5'-dithiobis-(2-nitrobenzoic acid)

acid composition, 72, 7 and 2 cysteines/mol for wheat (C_3) RuBPCase and its large and small subunits, respectively

Titration of sulfhydryl groups of activated maize RuBPCase was accompanied by a concomitant loss of enzyme activity (Fig 4); therefore, some of the sulfhydryl groups of RuBPCase must be involved in the catalysis of

Table 3 Sulfhydryl group content of maize leaf RuBPCase

Enzyme or subunit	Pre-treatment	Sulfhydryl group/ mol protein*
Holoenzyme	Control	76
	1% SDS	78
	10 mM HCO_3^- + 10 mM Mg^{2+}	72
Large subunit	1% SDS	8
Small subunit	1% SDS	2

*Sulfhydryl group content was determined by reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) at pH 8 0

the enzyme. This conclusion is consistent with the results of Takabe and Akazawa [26] using DTNB for sulfhydryl group titration and of recent studies using affinity labelling with C_3 RuBPCase [28, 29]

Amino acid composition

The amino acid composition of maize RuBPCase holoenzyme and subunits is presented in Table 4 The total amino acid residues for the native enzyme and its LSU and SSU were 4843, 494 and 112, respectively In general, the amino acid composition (g amino acids/100 g recovered amino acids) of the LSU reflected the amino acid composition of the native enzyme This is to be expected since the contribution of the LSU to the native enzyme is so great (80%) However, there are notable differences in the amino acid compositions of the LSU and SSU The SSU had less (< 20%) His, Arg, Gly, Ala and Ile than the LSU while it contained more (> 20%) Pro, Tyr, Phe and Trp

McIntosh *et al* [30] deduced the amino acid sequence of the LSU of maize RuBPCase from the nucleotide sequence of the structural region of the LSU gene and concluded that the LSU polypeptide consists of 475 amino acid residues and has a MW of 52 682 Our data for amino acid residues per LSU polypeptide differ by only 4%, 494 vs 475, and our data for LSU MW differ by only 1 5%, 53 500 vs 52 700

Yeoh *et al* [19] reported that the total amino acid residues for native RuBPCase of wheat and its LSU and SSU were 4780, 481 and 117, respectively They concluded that the overall amino acid pattern of wheat RuBPCase was similar to those for barley, oat, spinach, beet and tobacco [17] However, among these C_3 species the data from the Gramineae species more closely resembled each other than they did the more distantly related dicots. Specifically, the enzyme from Gramineae species had lower Thr, Val, Leu and Tyr but higher Pro content [19] Extending their comparison to native RuBPCase from maize, however, we find that the general statement made for Gramineae species above is valid for only Val and Leu Maize RuBPCase contains as much Thr as the dicots, more Tyr than the other monocots but slightly less than the dicots, and the least Pro content of both these monocots and dicots In general, the amino acid composition of maize RuBPCase and its subunits revealed no distinguishing feature between C_3 and C_4 Gramineae species nor between them and the dicots mentioned

DISCUSSION

The purified RuBPCase from the C_4 plant maize is similar to the enzyme of many C_3 plants, including monocots and dicots, with respect to specific activity, MW of the holoenzyme and its subunits, sedimentation coefficient and amino acid composition However, polypeptide composition and apparent isoelectric point of maize RuBPCase are slightly different from those reported for the C_3 plant enzyme. All available evidence indicates that C_4 plants have evolved from C_3 plants independently many times in different taxonomic groups although the precise evolutionary transition sequence from C_3 to C_4 is not clear [31] It has been reported that the enzyme from C_3 and CAM plants has a lower K_m (CO_2) value than C_4 plants [12, 13] If this represents an evolutionary change in function of the enzyme, it then appears that only a minor change such as changes in amino acid sequence of a small portion of LSU [23] can

Table 4 Amino acid composition* of maize RuBPCase and subunits

Amino acid	Native enzyme (MW 532 000)		Large subunit (MW 53 500)		Small subunit (MW 13 000)	
Lys	6 75	(280)	6 77	(28)	7 09	(7)
His	3 11	(121)	3 74	(15)	1 24	(1)
Arg	6 91	(235)	7 48	(26)	5 00	(4)
Asx	9 40	(435)	9 69	(45)	10 22	(11)
Thr	5 78	(304)	6 03	(32)	4 98	(6)
Ser	3 93	(240)	3 71	(23)	5 07	(7)
Glx	11 08	(460)	10 77	(45)	10 94	(11)
Pro	4 39	(240)	3 55	(20)	5 99	(8)
Gly	5 56	(519)	6 17	(58)	4 03	(9)
Ala	5 99	(448)	6 69	(50)	3 80	(7)
Cys†	1 44	(74)	1 42	(7)	1 59	(2)
Val	5 30	(285)	5 29	(29)	4 94	(6)
Met	2 28	(93)	2 40	(10)	2 20	(2)
Ile	4 46	(210)	4 78	(23)	3 73	(4)
Leu	8 26	(388)	8 25	(39)	8 58	(10)
Tyr	5 91	(193)	4 85	(16)	9 59	(8)
Phe	6 35	(230)	5 61	(20)	6 72	(6)
Trp‡	3 10	(88)	2 80	(8)	4 29	(3)
Total	100 00	(4843)	100 00	(494)	100 00	(112)

*Results expressed as g amino acid/100 g recovered amino acid Numbers in parentheses represent amino acid residues/mol protein

†Values determined by titration of sulfhydryl groups with DTNB

‡Data adopted from Yeoh *et al* [19]

bring about changes in kinetic properties. Comparisons of the properties of RuBPCase from maize, obtained in this study, with those of the C_3 enzyme in earlier studies suggests that RUBPCase was highly conserved during evolution.

EXPERIMENTAL

Extraction and purification of enzymes. All stages of enzyme preparation were carried out at 0–4°. Freshly harvested leaves (250 g) from 16-day-old maize (*Zea mays* L, FR9°ms × FR 37, Illinois Foundation Seeds) seedlings were cut into transverse segments (*ca* 5 mm) and dropped into a Waring commercial blender containing 1 l homogenizing medium of Yeoh *et al.* [19] plus 2% insoluble PVP Five 1-min blends at maximum speed were used to grind the tissue The homogenate was filtered through four layers of cheesecloth and then centrifuged at 27 300 *g* for 30 min The supernatant was filtered through two layers of Miracloth prior to $(NH_4)_2SO_4$ fractionation The ppt from the 35–60% $(NH_4)_2SO_4$ satd fraction was dissolved in 50 mM HEPES (pH 7.8) containing 1 mM EDTA·Na_2, and 1 mM DTT [19] and then centrifuged at 100 000 *g* for 2 5 hr in a Beckman 30 rotor The supernatant was filtered through Miracloth and 10-ml aliquots were carefully pipetted into 95 × 14 mm cellulose nitrate centrifuge tubes containing 2 ml 35% (w/v) sucrose in HEPES buffer soln satd to 60% $(NH_4)_2SO_4$ and then overlayered with 1 ml 35% sucrose in HEPES buffer soln. Centrifugation was carried out at 190 000 *g* for 17 hr in a Beckman SW-40 rotor The pellets were dissolved in 25 mM Tris–HCl (pH 7.5), containing 25 mM KCl and 5 mM DTT and dialysed for 2 hr against 2 l of the same buffer soln. Aliquots of the dialysed fraction were layered onto 36 ml linear sucrose density gradients, 0.29–0 84 M sucrose in the Tris buffer soln containing 25 mM $MgCl_2$ as well as KCl and DTT [32] and centrifuged at 112 700 *g* for 40 hr in a Beckman SW-27 rotor 1 ml fractions were collected and assayed for RuBPCase and PEP carboxylase enzyme activities. Active RuBPCase fractions free of PEP carboxylase activity were combined and ppted with $(NH_4)_2SO_4$ at 60% satn. The pellet was dissolved in 20 mM Tris–HCl (pH 8.0), containing 1 mM $MgCl_2$, and 1 mM DTT and subjected to gel filtration on Sepharose 6-B (93.5 × 2.8 cm) equilibrated with the same buffer soln [33] *A* at 280 nm was monitored and 5 ml fractions were collected. Peak tubes were combined, the $A_{280/260}$ ratio and protein were determined and the protein ppted with $(NH_4)_2SO_4$ at 60% satn. The purified enzyme was stored at −80° as a ppt in the 60% satd $(NH_4)_2SO_4$ after making the mixture 100 mM DTT, purging with N_2 and freezing in liquid N_2 [34]. Determinations of total and specific enzyme activity and protein were made at each stage of purification. Protein was determined by the Lowry method [35] and/or the Bradford method [36] using ovalbumin as the standard

Enzyme assays. RuBPCase was assayed according to Lorimer *et al.* [37]. PEP carboxylase was assayed spectrophotometrically by measuring the oxidation of NADH at A_{340} coupled to malate dehydrogenase [38].

Separation of subunits. The enzyme was dialysed overnight at 4° against 2 l 50 mM Tris–HCl (pH 8.5) containing 1 mM EDTA·Na_2 and then carboxymethylated according to Kung *et al.* [21] The subunits were separated in darkness at room temp on a Sephadex G-100 column (88 5 × 3 5 cm) equilibrated with 50 mM Tris–HCl (pH 8.5), 1 mM EDTA·Na_2, and freshly prepared 8 M urea. The fresh urea buffer soln was degassed and passed over a bed of amberlite MB-1 immediately before use to remove any cyanate and thus avoid carbamylation. Fractions corresponding to the large and the small subunits, according to A_{280} monitoring, were combined and subjected to slow dialysis against 50 mM Tris–HCl (pH 8.5). Dialysis was initially done at room temp but changed to 4° about halfway through the procedure Following dialysis, the subunits were concd, using a UM-20 Amicon filter for the large subunit and a UM-2 Amicon filter for the small subunit, and protein was determined Subunits were stored at −80° as $(NH_4)_2SO_4$ ppts at 60% satn.

Sample preparation for polyacrylamide gel electrophoresis. Freshly obtained holoenzyme or subunits were divided into aliquots and stored at −80°. Samples prepared for electrophoresis under non-denaturing conditions typically contained 2–5 mg protein/ml plus 5% β-mercaptoethanol and 5% glycerol A range from 10 to 50 μg protein was loaded into each gel slot to ascertain the degree of purity.

Samples analysed by SDS 1D-electrophoresis had 2% SDS which was generally included in the above mixture. Samples were heated at 100° for 3 min just prior to electrophoresis. From 2–10 μg isolated subunits were loaded into each gel slot MW marker proteins were run simultaneously using a premixed soln according to Ames [39]. The proteins used and their subunit MWs were· rabbit muscle phosphorylase *a* (94 000), human transferrin (80 000), bovine serum albumin (68 000), ovalbumin (43 000), horse liver alcohol dehydrogenase (37 000), beef heart lactic dehydrogenase (30 500), bovine milk β-lactoglobulin (18 400) and egg white lysozyme (14 300)

Samples prepared for isoelectric focusing typically contained 5–10 mg protein/ml and included 0.5% SDS, 8 M urea and 5% β-mercaptoethanol. From 2–50 μg protein of purified holoenzyme was loaded on the 1D-slab isoelectric focusing gel, while 10–15 μg protein was loaded onto cylindrical isoelectric focusing gels.

Polyacrylamide gel electrophoresis 1D-gel electrophoresis under non-denaturing conditions was performed on 0 75 mm thick slab gels using a modification of the Laemmli gel system [40]. Protein samples were stacked electrophoretically in a 4% acrylamide gel containing 62 5 mM Tris–HCl, pH 6 8, and resolved in a 6% acrylamide gel containing 187 5 mM Tris–HCl, pH 8 8. The electrode buffer contained 192 mM glycine, 25 mM Tris–Base and 5 mM cysteine–HCl. Electrophoresis was performed with a constant current of 15 mA/slab for 1.5–2 hr

1D-electrophoresis in the presence of SDS was performed on gels containing 14% acrylamide and 0 4% *N,N'*-methylenebisacrylamide using the discontinuous buffer system of Laemmli [40] Electrophoresis was performed with a constant current of 20 mA/slab for 2–2 5 hr

2D-gel electrophoresis with isoelectric focusing in the first dimension and SDS in the second dimension was performed as described by O'Farrell [41], but with 0.4% pH 3 5–10 and 1 6% pH 5–7 ampholytes SDS was included in the samples at 0 5% to ensure complete dissociation of the holoenzyme into subunits Isoelectric focusing was typically conducted for a total of 6400 V-hr (14 hr at 400 V, 1 hr at 800 V) SDS slab gels used for the second dimension were prepared identically to 1D-gels. Slab gels were stained with 0 1% Coomassie brilliant blue R in 45% TCA for 20–25 min. Gels were destained with several changes of 7% HOAc. Slab gels were photographed using Panatomic-X film and then dried down onto Whatman 3 MM paper under vacuum [42].

1D-isoelectric focusing for analysis of isoenzyme subunits was performed on 0 75 mm thick polyacrylamide slab gels, having the same composition as the gel described by O'Farrell [41] for isoelectric focusing in cylindrical tubes The ampholyte composition was 1% pH 3 5–10 and 1% pH 5–7 Isoelectric focusing was conducted identically to that used in 2D-gel electrophoresis. Slab gels were washed extensively in 30% MeOH and 7% HOAc and then stained and destained as described above

Non-denaturing preparative isoelectric focusing Ca 25 mg of partially purified enzyme samples [35–55% satd $(NH_4)_2SO_4$

fraction] were dialysed against 1% glycine for 5 hr and then loaded with a sample applicator as a zone onto the gel bed The granulated gel contained 6 5% prewashed Sephadex G-75 Superfine and 2% LKB Ampholine Ampholytes (pH range 3 5-10) Electric focusing was carried out for 16 hr at 8 W constant power and 10° After focusing was completed the gel was separated by pressing the fractionating grid through the gel bed and the pH gradient measured with the aid of a surface glass electrode The separated zones were collected by sectioning the gel bed with the fractionating grid and then transferring to gel-supporting columns To elute the proteins two gel vols of buffer were added to the columns Fractions were assayed immediately after elution for protein content and PEP carboxylase and RuBPCase activities

Titration of free sulfhydryl groups Titration of native RuBPCase and subunits with DTNB was carried out at 25° in 0 1 M Tris-SO_4 buffer (pH 8 0) in a final vol of 1 0 ml [43] For titration of fully activated enzyme the native enzyme was preincubated with 10 mM $NaHCO_3$ and 10 mM $MgCl_2$ for 30 min at 25 prior to titration For titration under denaturing conditions a soln of native RuBPCase or subunits was made 1% with respect to SDS, heated at 100° for 2 min, cooled to 25° and then titrated The reaction was monitored continuously at 412 nm, i e increase in A due to the liberation of 2-nitro-5-thiobenzoate ($E_{412} = 13\,600\,M^{-1}\,cm^{-1}$) A values were corrected for A due to DTNB (minus protein, ±SDS) Purified maize RuBPCase and its subunits had negligible A at 412 nm

Amino acid analyses Aliquots of the holoenzyme and subunits were hydrolysed in 6 N HCl by reflux for 20 hr under N_2 The hydrolysates were evaporated to dryness under vacuum using a rotary evaporator and washed twice with H_2O The residues were dissolved in pH 2 2 diluting buffer containing 0 5 μmol/ml α-amino-β-guanidinopropionic acid and norleucine as int standards Amino acid analyses were performed on a Beckman Model 121 automatic amino acid analyser with a column temp of 54° Separations were achieved using Beckman Type W-1 resins in both the long and short columns and pH 5 25, 3 25 and 4 25 buffer systems [44]

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