AN UNUSUAL LARGE SUBUNIT MULTIPLE POLYPEPTIDE COMPOSITION OF PERENNIAL RYEGRASS RIBULOSEBISPHOSPHATE CARBOXYLASE/OXYGENASE

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1. Introduction

Chloroplast DNA (ctDNA) has the capacity to encode for as many as 100 average-sized proteins, although to date only a few of the chloroplastic products have been identified [1]. The most thoroughly investigated of the ctDNA-encoded polypeptides is the large, catalytic subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBPCase, EC 4.1.1.39). RuBPCase is a bifunctional enzyme which catalyzes the initial reaction in each of two opposed metabolic pathways, the C_3 photosynthetic carbon reduction and the C_2 photorespiratory carbon oxidation cycles [2]. The higher plant-type enzyme is a hexadecamer ($M_r \approx 550\,000$) composed of 8 catalytic subunits ($M_r \approx 55\,000$) and 8 small, non-catalytic subunits ($M_r \approx 14\,000$). Since the large subunit is encoded on ctDNA, it is maternally inherited and synthesized in the chloroplast [3,4]. In contrast, the small subunit, or more precisely the 20 000 M_r small subunit precursor polypeptide, is encoded by nuclear DNA, synthesized in the cytoplasm and exhibits Mendelian inheritance [4,5]. The precursor polypeptide is transported across the chloroplast envelope membranes, processed in the stroma and ultimately assembled with large subunits to form the holoenzyme [4]. Further structural complexity is revealed by polyacrylamide slab gel isoelectric focusing of the dissociated, carboxymethylated holoenzyme in the presence of 8 M urea. Under these conditions the large subunit is resolved into 3 major polypeptides which cluster near pH 6.0 and which are separated from each other by ~0.1 pH units [6]. Comparative studies of the RuBPCase polypeptide composition from a variety of higher plants have revealed that the presence of 3 large subunit polypeptides is a characteristic structural feature of the protein [7–13]. In contrast, the small subunit separates into 1–4 major polypeptides (which cluster near pH 5.0) depending on the plant species from which the RuBPCase under investigation is isolated [6–13]. Although the precise nature of the large and small subunit polymorphism is not known, this multiple polypeptide composition has been extensively used to investigate evolutionary relationship within several plant genera including *Nicotiana* [8], *Lycopersicon* [9], *Avena* [10], *Solanum* [11], *Triticum* [12] and *Brassica* [13].

Here, we report the presence of 5–6 major large subunit polypeptides in the enzyme isolated from diploid and tetraploid cultivars of perennial ryegrass (*Lolium perenne* L.). This is the first reported exception to the 3 large subunit polypeptide structure of higher plant-type RuBPCase.

2. Materials and methods

Seeds and tillers of diploid ($2n = 2X = 14$) (cvs. Gremie and 64038-50308) and tetraploid ($2n = 4X = 28$) (cv. Reveille) cultivars of *Lolium perenne* L. were kindly provided by Dr M. K. Garrett, The Queen's University of Belfast. The plants were grown in a controlled environment room (800 $\mu E \cdot m^{-2} \cdot s^{-1}$, 16 h photoperiod, 21°C day/16°C night) and ploidy level was verified by chromosome counts using root-tip smears.

Ryegrass RuBPCase was purified at 4°C from young, fully expanded leaves by 3 different protocols. The first method involved $(NH_4)_2SO_4$ fractionation (35–55% satn., 4°C) of the crude leaf extract and repeated gel filtration through Sepharose 6B [14].

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The second protocol was similar except that the 30–55% satn. $(NH_4)_2SO_4$ fraction was desalted on Sephadex G-25, layered onto discontinuous 0.2–0.8 M sucrose gradients and centrifuged for 18 h at 27 000 rev./min (96 300 × g, r_{av}) in a Beckman SW 27 rotor [15]. Ryegrass RuBPCase was also obtained as crystals from crude leaf extracts using polyethylene glycol 6000 as the precipitant (S. J., R. C., unpublished). Crystalline tobacco (*Nicotiana tabacum* L. cv. Xanthi) carboxylase was isolated from crude leaf extracts and recrystallized twice before use [16]. RuBPCase was purified from market spinach (*Spinacia oleracea* L.) leaves exactly as in [17]. The proteins isolated by these various methods were adjudged to be homogeneous by nondenaturing electrophoresis in 4% polyacrylamide tube gels [15] and by SDS electrophoresis in 12% polyacrylamide slab gels (SDS–PAGE) [18]. Ryegrass RuBPCase purified by any of the 3 techniques had a specific carboxylase activity of ~1 μmol $H^{14}Co_3^-$ fixed . min^{-1} . mg $protein^{-1}$ at 30°C. The spinach and tobacco carboxylases possessed specific activities comparable to those in [15–17].

Subunit multiple polypeptide composition was examined by isoelectric focusing in the presence of 8 M urea (urea–IEF) using dissociated, carboxymethylated protein. Preliminary studies used RuBPCase samples carboxymethylated as in [19]. However, using this protocol we occasionally detected spurious bands with the homogeneous spinach and tobacco preparations which we attributed to non-specific iodoacetic acid side reactions upon brief exposure to oxygen during gel filtration. Therefore, we have modified the protocol in [19] such that after the carboxymethylation reaction had continued for 10 min under N_2 at 25°C in the dark, a 100-fold molar excess of 2-mercaptoethanol was injected to quench the reaction and the protein solution was gel filtered through a Sephadex G-25 column equilibrated with unbuffered 8 M urea. The dissociated, carboxymethylated proteins (20–35 μg) were immediately applied to a prefocused 5% polyacrylamide slab gel containing 8 M urea and 2% (w/v) Pharmalyte (pH 4–6.5); Pharmalyte (pH 5–8); or LKB (pH 5–8) carrier ampholyte. Electrofocusing was as in [19] at 300 V for 18 h at 4°C. The gel was then removed from the plates, fixed for 1 h in 4% sulfosalicylic acid/12.5% trichloroacetic acid and washed for 10–15 min in 40% methanol/10% acetic acid. The subunit bands were visualized by staining the gel for 1 h in 40% methanol/10% acetic acid containing 0.1% Coomassie brilliant blue R-250 and by destaining in 20% methanol/10% acetic acid.

The first dimension of the 2-dimensional urea–IEF/SDS–PAGE system was performed as above except that electrofocusing of the carboxymethylated, urea-dissociated protein was done in tube gels. Before SDS–PAGE in the second dimension, the focused gels were soaked in 3 consecutive changes of SDS–equilibration buffer (30 min, each) [20]. The 12% polyacrylamide SDS–slab gel was prepared according to [18] and run at 20 mA until the tracking dye was within 1 cm from the bottom. The gel was fixed and stained in 40% methanol/10% acetic acid containing 0.1% Coomassie brilliant blue and destained as above.

3. Results and discussion

Non-denaturing polyacrylamide gel electrophoresis in 4% tube gels showed that the native diploid and tetraploid ryegrass RuBPCases comigrate and run in a manner completely analogous to the spinach and tobacco enzymes. Similarly, SDS–PAGE failed to detect any M_r differences between the ryegrass, spinach and tobacco large or small subunits [14]. The large subunit M_r was estimated to be ~54 500 and that of the small subunit ~13 000. Contrary to [21], we found no evidence to suggest that the ryegrass RuBPCase small subunit has an unusually high M_r (≈18 000). In summary, spinach, tobacco and the 2 × and 4 × ryegrass carboxylases do not differ significantly in any of these electrophoretic properties.

Isoelectric focusing of dissociated, carboxymethylated spinach RuBPCase in the presence of 8 M urea revealed 3 major, equally spaced large subunit polypeptides and 2 small subunit polypeptides (fig.1B). The carboxymethylated tobacco protein displayed a similar multiple polypeptide composition, although the precise locations of the large and small subunit polypeptides in the pH gradient differed from those of the spinach carboxylase (fig.1A,B). These comparative results with the spinach and tobacco enzymes are in excellent agreement with [7,8,19,22]. Similarly, urea–IEF analysis of RuBPCase isolated from *Moricandia arvensis*, a crucifer, revealed the presence of 3 large subunit polypeptides and 3 small subunit polypeptides (S. J., R. C., unpublished). This is the first reported focusing of the *M. arvensis* enzyme, and as with all other higher plant-type RuBPCases [7] the presence of 3 major large subunit polypeptides is a characteristic structural feature of the protein. However, the dissociated, carboxymethylated 2 ×

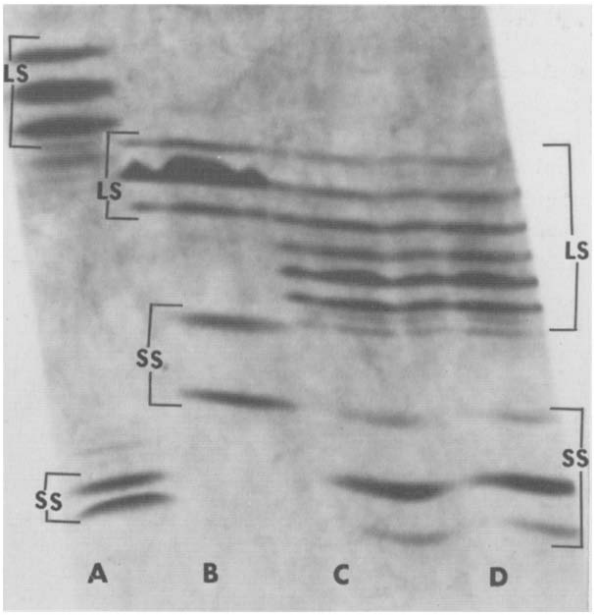

Fig.1. Multiple polypeptide composition of dissociated, carboxymethylated RuBPCase (25 μg, each) from (A) tobacco, (B) spinach, (C) 2 × (cv. Gremie) ryegrass and (D) 4 × ryegrass as revealed by isoelectric focusing in a 5% polyacrylamide slab gel containing 8 M urea and 2% Pharmalyte (pH 4–6.5) ampholyte. LS and SS are large and small subunit polypeptides, respectively.

and 4 × ryegrass RuBPCases differed significantly in large subunit multiple polypeptide composition from the tobacco, spinach (fig.1) and *M. arvensis* enzymes and all other higher plant-type carboxylases examined to date [6–13,19,22]. The large subunits of the ryegrass enzymes were composed of 5–6 major polypeptides and the small subunits consisted of 3 major polypeptide bands (fig.1C,D). The ryegrass catalytic subunit polypeptides were equally spaced ~0.1 pH units apart. Furthermore, the large subunit polypeptides of the carboxymethylated ryegrass, spinach and tobacco RuBPCases all clustered in the same region of the pH gradient under these conditions. Neither the isolation protocol for the 2 × and 4 × ryegrass enzymes (i.e., purification by gel filtration, sucrose density gradient centrifugation or crystallization), the presence or absence of a variety of protectants during tissue homogenization (e.g., dithiothreitol, Na-metabisulfite, Dowex 1–X10, insoluble polyvinylpolypyrrolidone [22]) nor the choice of carrier ampholyte (Pharmalyte pH 4–6.5 and pH 5–8 or LKB pH 5–8) had any effect on the multiplicity of the ryegrass large subunit polypeptides. Similarly, when tobacco and 4 × ryegrass leaf tissue was combined, homogenized and RuBPCase purified directly by polyethylene glycol 6000-induced crystallization, the expected composite (cf. fig.1A,D) of 9 large subunit and 4 small subunit polypeptides was obtained. These observations, together with the predicted focusing patterns obtained with the dissociated, carboxymethylated spinach, tobacco (fig.1) and *M. arvensis* proteins (cf. [7]) under identical conditions, minimize the possibility that the unusual multiplicity of large subunit polypeptides observed with the 2 × and 4 × ryegrass RuBPCases is an artifact of our extraction, purification, carboxymethylation or urea–IEF protocols.

Due to the variable number [1–4] of small subunit polypeptides between different higher plant RuBPCases [7], the possibility that the ryegrass enzymes may possess several relatively less acidic small subunit polypeptides which focus in the more basic large subunit region of the pH gradient (cf. [23]) was examined. Two-dimensional urea–IEF/SDS–PAGE analysis of the carboxymethylated ryegrass proteins revealed that the 5–6 major polypeptides in the large subunit region of the focusing gel each have M_r ~54 000 (fig.2), indicative of a large subunit polypep-

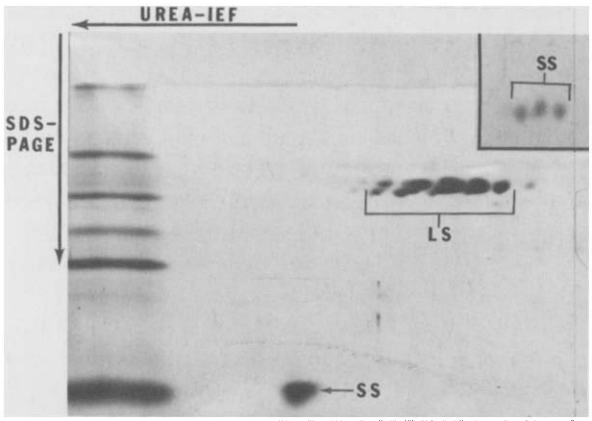

Fig.2. Two-dimensional urea–IEF/SDS–PAGE analysis of 4 × ryegrass RuBPCase. The dissociated, carboxymethylated protein (35 μg) was first electrofocused in a tube gel as in fig.1 and then subjected to SDS electrophoresis in a 12% polyacrylamide slab gel. The left lane contains the following protein M_r standards (7 μg, each) in order of increasing electrophoretic mobility: bovine serum albumin (68 000); fumarase subunit (48 500); aldolase subunit (40 000); carbonic anhydrase (29 500); and cytochrome *c* (12 500). The inset shows the 3 small subunit polypeptides better resolved in a separate, but identical, 2-dimensional gel. LS and SS are large and small subunit polypeptides, respectively.

tide. Similarly, the 3 putative small subunit polypeptides of ryegrass RuBPCase comigrate in the second dimension (fig.2, inset), each having M_r ~13 000 (fig.2). Comparative analysis of the dissociated, carboxymethylated tobacco protein by 2-dimensional urea–IEF/SDS–PAGE revealed 3 large subunit polypeptides ($M_r \approx 54\,000$) and 2 small subunit polypeptides ($M_r \approx 13\,000$).

4. Concluding remarks

Our rationale for investigating ryegrass RuBPCase was the intriguing report [21] that the purified 2 X and 4 X enzymes differed in their relative affinities for the gaseous substrate CO_2. The molecular basis of this difference was ascribed to a charge shift of ~0.3 pH units between the native diploid and tetraploid isozymes [21]. Since the differences in $K_m(CO_2)$ and isoelectric point were consistently observed with both non-isogenic and isogenic cultivars of perennial ryegrass, it was concluded that ploidy level (i.e., nuclear gene dosage) per se was responsible for this genetically-controlled variation in RuBPCase [21]. To date, we have been unable to detect any kinetic or structural (e.g., fig.1) differences between the homogeneous 2 X and 4 X enzymes [14]. Although our findings disagree with [21], we have discovered that both the diploid and tetraploid ryegrass RuBPCases do differ with respect to the multiple polypeptide composition of the large, catalytic subunits when compared to all other higher plant-type carboxylases examined. One-dimensional isoelectric focusing of the dissociated, carboxymethylated ryegrass enzymes in the presence of 8 M urea and 2-dimensional urea–IEF/SDS–PAGE analysis reproducibly resolve 5–6 major large subunit polypeptides ($M_r \approx 54\,000$) and 3 small subunit polypeptides ($M_r \approx 13\,000$) (fig.1,2). The presence of 5–6 large subunit polypeptides in both the 2 X and 4 X ryegrass RuBPCases is very unusual from a comparative point of view since extensive studies with diverse higher plant-type carboxylases have always resolved the denatured, carboxymethylated protein into 3 major large subunit polypeptides upon urea–IEF [6–13,19,22]. The only reported exceptions to this general feature of RuBPCase subunit structure have been the proteins isolated [24,25] from 2 different interspecific parasexual hybrids in the genus *Nicotiana*. The unusual multiplicity of large subunit polypeptides in these leaf protoplast fusion hybrids was attributed to the expression of both sets of ctDNAs. Whether this suggests that, by analogy, perennial ryegrass possesses a mixture of 2 chloroplast types (i.e., biparental inheritance of ctDNA) or that this species has some other unique molecular processes affecting RuBPCase large subunit structure (e.g., post-translational and/or post-transcriptional modifications) awaits further investigation.

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