SUBUNIT STOICHIOMETRY OF TOBACCO RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE

J. C. GRAY, E. A. HOOPER† and R. N. PERHAM†
Departments of Botany and †Biochemistry, University of Cambridge, Cambridge CB2 3EA, England

Received 2 April 1980

1. Introduction

Ribulose 1,5-bisphosphate carboxylase (EC 4.1.1.39) is an oligomeric protein, with mol. wt ~550 000, made up of large subunits (L) with mol. wt 55 000 and small subunits (S) with mol. wt ~13 000 [1,2]. There is reasonable chemical and physical evidence for the presence of 8 large subunits in the molecule [2–4] but unequivocal experimental evidence for the number of small subunits is lacking. X-ray diffraction and electron microscopy of form I crystals of the enzyme from tobacco indicated that each subunit must be present in a multiple of 4 copies but, because of the low molecular weight of the small subunit and the spread of molecular weights estimated for the enzyme (475 000–600 000 [1]), it was not possible to distinguish between molecules of stoichiometry L_8S_8, L_8S_{12} or L_8S_{16} [4]. The stoichiometry L_8S_{12} was eliminated when form II crystals of the tobacco enzyme where shown to possess D_4 (422) symmetry [5]. However, when arguments about possible self-assembly mechanisms are excluded, the crystal structure does not yet allow an unequivocal determination of the subunit stoichiometry, although the crystal density of form III crystals supports the stoichiometry L_8S_8 [6].

In the absence of any reliable chemical data on the number of small subunits in the protein, we have used the amidination method in [7] to determine the subunit stoichiometry of tobacco ribulose bisphosphate carboxylase. The results indicate the presence of equal numbers of large and small subunits in the protein and support a stoichiometry of L_8S_8.

2. Materials and methods

2.1. *Protein*

Crystalline (form I) ribulose 1,5-bisphosphate carboxylase was prepared from young tobacco leaves by the method in [8], as modified [9]. The protein was recrystallised 3 times. For the isolation of subunits, the protein (50 mg) was dissolved in 2 ml 25 mM Tris–HCl (pH 7.8), 0.5 mM EDTA, 5% sodium dodecylsulphate (SDS), 100 mM 2-mercaptoethanol and heated at 100°C for 2 min. The subunits were separated by gel filtration on a 95 × 2.35 cm column of Sephadex G-200 in 25 mM Tris–HCl (pH 7.8), 0.5 mM EDTA, 0.5% (w/v) SDS, dialysed against 0.5% (w/v) NH_4HCO_3 for 72 h and lyophilised.

2.2. *Amino acid analysis*

Subunits (0.2 mg) were dissolved in 1.0 ml 6 M HCl and 10 μl 2-mercaptoethanol and hydrolysed for 24, 48 and 72 h at 105°C in sealed evacuated tubes. Hydrolysates were analysed on a Rank Hilger Chromaspek amino acid analyser coupled with a Digico micro 16 V computer. Tryptophan content was determined by UV spectroscopy of subunits dissolved in 0.1 M NaOH [9]. Cysteine content was determined by titration with 5,5′-dithiobis (2-nitrobenzoic acid) in the presence of 8 M urea [11].

2.3. *Amidination*

The enzyme was amidinated by methyl [1-^{14}C]-acetimidate following [7] as modified [11]. Freeze-dried protein (0.5 mg) was treated with 10 μmol methylacetimidate in 0.1 ml 0.2 M sodium borate

buffer (pH 10.0) containing 6 M guanidine–HCl, 2 mM EDTA and 0.01% (w/v) sodium azide, and the reaction allowed to proceed for 4 h at 20°C. Samples were dialysed against 20 mM sodium phosphate buffer (pH 7.0) containing 2 mM EDTA, 0.1% (w/v) SDS and 0.01% (w/v) sodium azide, lyophilised and analysed by SDS–polyacrylamide gel electrophoresis [7]. Radioactivity in the subunits bands was determined as in [7].

3. Results and discussion

The determination of subunit ratios in a protein by the amidination method requires a knowledge of the content of lysine residues in the subunits. The published amino acid analyses of the subunits of the tobacco enzyme show some variation [9,12,13] so a full amino acid analysis was carried out on subunits separated by gel filtration on SDS–Sephadex G-200. SDS–Polyacrylamide gel electrophoresis indicated that the subunit preparations were not contaminated with other polypeptides (fig.1A). The results of the amino acid analyses are shown in table 1. The lysine contents of the large and small subunits were 23.8 and 9.3 residues per subunit, respectively.

Amidination was carried out on two separate preparations of the enzyme crystallised from extracts of tobacco leaves as in [8]. SDS–Polyacrylamide gel electrophoresis of the crystalline protein is shown in fig.1B. The preparations contained in addition to the large subunit (M_r = 55 000) and small subunit (M_r = 13 000) a staining band with a mobility equivalent to a polypeptide of mol. wt 110 000. This has been identified as a dimer of the large subunit [14,15] and radioactivity incorporated into this band was included with the radioactivity in the large subunit band. The large subunit dimer accounted for ~7% of the radioactivity incorporated into the large subunit. The results of the amidination experiments are presented in table 2. The mean ratio of radioactivity in the two subunits (L:S) was 2.58 ± 0.13, which gives a subunit stoichiometry (L:S) of 1.00 ± 0.01, calculated from the value of 2.56 for the molar ratio of lysine residues in the large and small subunits.

Our experiments clearly establish the presence of equal numbers of large and small subunits in the structure of ribulose bisphosphate carboxylase. This stoichiometry eliminates the subunit composition L_8S_{16} that was not excluded by the X-ray diffraction studies. A structure with the composition L_8S_8 is therefore the only one that can satisfy the crystal structure data and the results from the amidination experiments.

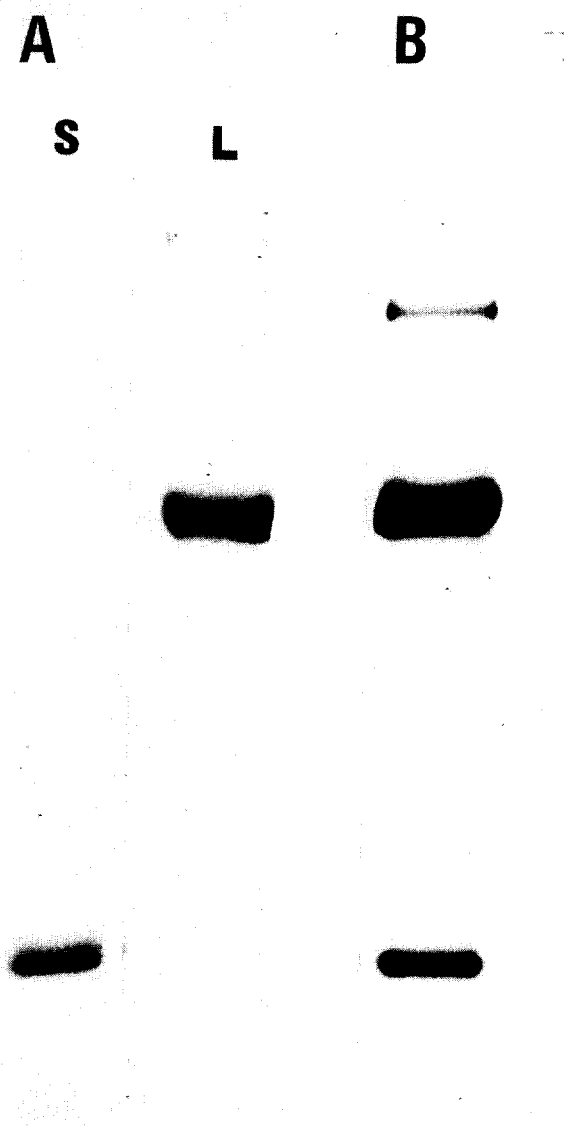

Fig.1. SDS–polyacrylamide gel electrophoresis of ribulose 1,5-bisphosphate carboxylase and its separated subunits. (A) Large (L) and small (S) subunits separated by gel filtration on SDS–Sephadex G-200. (B) Crystalline ribulose 1,5-bisphosphate carboxylase. The very faint band migrating more slowly than the large subunit dimer is an aggregation state of the large subunit that forms on storage. It was not present in freshly prepared enzyme or large subunit.

Table 1
Amino acid composition of subunits of tobacco ribulose 1,5-bisphosphate carboxylase

	Large subunit		Small subunit	
	mol %	residues/subunit	mol %	residues/subunit
Asp	9.68	47.0	9.17	10.4
Thr	7.16	34.7	5.21	6.0
Ser	3.70	18.0	4.89	5.6
Glu	9.31	45.3	14.09	16.1
Pro	3.93	19.1	5.44	6.2
Gly	11.46	55.8	8.58	9.8
Ala	10.05	48.8	5.35	6.1
Val	7.92	38.5	6.70	7.6
Met	1.69	8.2	1.43	1.6
Ile	3.33	16.2	3.24	3.7
Leu	9.10	44.2	9.33	10.6
Tyr	3.67	17.8	8.61	9.8
Phe	4.39	21.3	4.23	4.8
His	3.37	16.4	2.48	2.8
Lys	4.91	23.8	8.17	9.3
Arg	6.31	30.6	3.07	3.5
Cys	–	9.0	–	3.0
Trp	–	7.2	–	4.0

Table 2
Subunit stoichiometry of tobacco ribulose 1,5-bisphosphate carboxylase

Protein sample	[^{14}C]Acetimidate labelling (dpm) L:S	Stoichiometry L:S
I	2.52 ± 0.12 (4)	0.98 ± 0.02
II	2.62 ± 0.12 (6)	1.02 ± 0.02

Acknowledgements

We thank Graham Parr for carrying out the amino acid analyses, and the Science Research Council and the Wellcome Trust for financial support.

References

[1] Kawashima, N. and Wildman, S. G. (1970) Ann. Rev. Plant Physiol. 21, 325–358.
[2] Rutner, A. C. (1970) Biochem. Biophys. Res. Commun. 39, 923–929.
[3] Wishnick, M., Lane, M. D. and Scrutton, M. C. (1970) J. Biol. Chem. 245, 4939–4947.
[4] Baker, T. S., Eisenberg, D., Eiserling, F. A. and Weissman, L. (1975) J. Mol. Biol. 91, 391–399.
[5] Baker, T. S., Eisenberg, D. and Eiserling, F. A. (1977) Science 196, 293–295.
[6] Baker, T. S., Suh, S. W. and Eisenberg, D. (1977) Proc. Natl. Acad. Sci. USA 74, 1037–1041.
[7] Bates, D. L., Harrison, R. A. and Perham, R. N. (1975) FEBS Lett. 60, 427–430.
[8] Chan, P. H., Sakano, K., Singh, S. and Wildman, S. G. (1972) Science 176, 1145–1146.
[9] Gray, J. C., Kung, S. D. and Wildman, S. G. (1978) Arch. Biochem. Biophys. 185, 272–281.
[10] Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70–77.
[11] Hale, G., Hooper, E. A. and Perham, R. N. (1979) Biochem J. 177, 136–137.
[12] Kawashima, N. (1969) Plant Cell Physiol. 10, 31–40.
[13] Kawashima, N., Kwok, S. Y. and Wildman, S. G. (1971) Biochim. Biophys. Acta 236, 578–586.
[14] Roy, H., Valeri, A., Pope, D. H., Rueckert, L. and Costa, K. A. (1979) Biochemistry 17, 665–668.
[15] Poulsen, C. (1979) Carlsberg Res. Commun. 44, 163–189.