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Ribulose-1,5-Bisphosphate Carboxylase as a Nuclear and Chloroplast Marker

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Summary. The data on the primary structure of ribulose-1,5-bisphosphate carboxylase/oxygenase are reviewed. Examples of their use as markers and in the elucidation of the evolution, adaptation and function of this key enzyme are given.

Key words: Fraction 1 protein – Amino acid sequences – Peptide maps

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Ribulose-1,5-bisphosphate carboxylate (E.C.4.1.1.39) in the chloroplasts of higher plants participates both in the photosynthetic carbon reduction cycle and in the photorespiratory carbon oxidation cycle (Lorimer, Woo, Berry and Osmond 1977). In the former, the enzyme catalyses the only reaction known to give a net increase in the amount of carbon compounds by fixing a molecule of CO_2 to generate 2 moles of glycerate-3-phosphate. In the latter, the enzyme acts as an oxygenase converting ribulose-1,5-bisphosphate into glycerate-3-phosphate and phosphoglycolate. The two moles of glycolate thus produced are converted in the peroxisomes and mitochondria into glycerate-3-phosphate with the release of a molecule of CO_2. The glycerate-3-phosphate can re-enter the carbon reduction cycle and the CO_2 is available for fixation by the carboxylase reaction. The flux of carbon through the linked photosynthetic reduction and the photorespiratory oxidation cycles is determined by the internal concentrations of CO_2 and O_2. The dual function of ribulose-bisphosphate carboxylase/oxygenase seems to protect the chloroplast against temporary CO_2 deprivation in the light: it permits the internal generation of CO_2, the dissipation of harmful photochemical energy through the production of glycolate by oxygenation and the partial recovery of the thus diverted carbon.

Sam Wildman, in his pioneering studies of the physical chemistry of leaf proteins, discovered this large molecular weight protein (550,000 dalton) that can account for up to 50 per cent of the soluble protein (fraction 1 protein) of a leaf. Since this enzyme is found in all photosynthetic organisms, it is the most abundant single protein species on earth.

Ribulose-1,5-bisphosphate carboxylase from higher plants and green algae can be dissociated into two types of subunits. Peptide mapping (Chan and Wildman 1972; Kawashima and Wildman 1972) and characterization by isoelectric focusing (Kung 1976, Sakano, Kung and Wildman 1974) of the subunits from *Nicotiana* species and their F_1 hybrids have shown that the large subunit (MW 55,000) is maternally inherited and that the small subunit (MW 12,000-15,000) is inherited in a Mendelian fashion.

The gene for the large subunit has been localized on chloroplast DNA in maize (Coen, Bedbrook, Bogorad and Rich 1977) and *Chlamydomonas* (Gelvin, Heizmann and Howell 1977). A restriction nuclease generated chloroplast DNA fragment from maize was cloned and directed the synthesis of the large subunit in an in vitro linked transcription-translation experiment. Messenger RNA for the large subunit has been isolated from *Chlamydomonas* polyribosomes with the aid of antibodies and was shown to hybridize with chloroplast DNA. The large subunit is synthesized on chloroplast ribosomes (Blair and Ellis 1973), whereas the small subunit is synthesized on cytoplasmic polyribosomes (Roy, Patterson and Jagendorf 1976) in a precursor form (Dobberstein, Blobel and Chua 1977; Highfield and Ellis 1978). The precursor form contains an additional amino acid sequence of 44 amino acids at the N-terminus (Chua, pers. commun.) which is cleaved off by an endoprotease in connection with its transfer across the chloroplast envelope.

In order to be useful as genetic markers, allelic differences at the level of the primary structure of the two subunits are desirable. In this paper the data on the primary structure of ribulose bisphosphate carboxylase are reviewed and examples of their use in the elucidation of the

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evolution, adaptation and function of this key enzyme are given.

As seen from Table 1, considerable allelic polymorphisms have been established by comparing the N-terminal sequence of the 110 to 120 amino acids of the small subunit in different species obtained by automatic Edman degradation (Gibbons, Strøbæk, Haslett and Boulter 1975; Haslett, Yarwood, Evans and Boulter 1976; Poulsen, Strøbæk and Haslett 1976; Holder, unpubl.).

Likewise, polymorphisms are apparent at the C-terminal end as determined by carboxypeptidase A digestion (Sugiyama and Akazawa 1970; Strøbæk, Gibbons, Haslett, Boulter and Wildman 1976; Poulsen 1977).

Spinach: -Phe-Leu-Thr-Tyr-COOH
Tobacco: -Thr-Val-Leu-Tyr-COOH
Barley: -Leu-Tyr-Phe-Val-Asn-Ala-COOH

The four additional amino acids on the C-terminal end of the small subunit in barley are consistent with its slightly higher molecular weight compared to the small subunits of spinach and tobacco. About 50 per cent of the total amino acid sequence of the small subunit in barley has been established by partial sequencing of three fragments obtained by cyanogen bromide cleavage (Poulsen, Strøbæk and Haslett 1976; Poulsen 1977).

Sequence information on the large subunit is so far only available for barley and spinach (Poulsen 1978; Stringer and Hartman 1978).

The large subunit of barley contains 9 methionine residues, which should yield up to 10 fragments after cyanogen bromide cleavage. Six fragments accounting for about 350 of the 490 residues have been partially sequenced after separation on a CM-cellulose column using a salt gradient in 8 M-urea and on a column of Bio Gel P-30. Two additional fragments have been recognized but not finally purified, one of them being N-terminally blocked. The N-terminal sequences of the characterized fragments are given in Table 2.

With the exception of one amino acid, the cyanogen bromide fragment CM 2-II from barley is identical in sequence to a tryptic peptide from spinach which was purified after reaction with 3-bromo-1,4-dihydroxy-2-butanone-1,4-bisphosphate as a substrate analogue affinity label (Stringer and Hartman 1978) and therefore this peptide is considered to be part of the catalytic site of the enzyme. The active site is known to be on the large subunit (Nishimura and Akazawa 1973).

The comparison of the corresponding sequences from barley and spinach have revealed four amino acid replacements among 35 analyzed residues. This definitively demonstrates species differences in the nucleotide sequence of the chloroplast gene coding for the large subunit of ribulose-bisphosphate carboxylase, and excludes a complete conservation of this ubiquitous protein.

The close cooperation of the nuclear and chloroplast genes in the biosynthesis of ribulose bisphosphate carboxylase has led us to investigate the sub-genus *Oenothera*, the evening primrose in which Otto Renner demonstrated that variegation, seedling lethality or embryo abortion of certain species hybrids were due to incompatibilities between the nuclear genome and the chloroplast

Table 1. N-terminal sequences of the small subunit of ribulose-1,5-bisphosphate carboxylase

					5					10					15					20					25
Oenothera biennis:	Phe	Asn	Val	Trp	Pro	Pro	Glu	Gly	Leu	Lys	Lys	Phe	Glu	Thr	Leu	Ser	Tyr	Leu	Pro	Pro	Leu	Thr	Arg	Glu	Gln
Barley :	Met	Gln				Ile			Ile													Ser	Thr		Ala
Pea :	Met	Gln					Ile		Lys								Trp						Pro	Asp	
Bean :	Met	Gln					Ile		Lys														Gln	Asp	
Tobacco :	Met	Gln					Ile Tyr	Asn Gly	Lys			Tyr								Asp		Ser	Gln	Gln	

Table 2. The N-terminal sequences of six cyanogen bromide fragments of the large subunit of ribulose,5-bisphosphate carboxylase in barley. Two fragments and one tryptic peptide from spinach are given for comparison.

Barley	CM-1	Pro-Ala-Leu-Thr-Glu-Ile-Phe-Gly-Asp-Asp-Ser-Val-Leu-Gln-Phe-Gly-Gly-Gly-Thr-Leu-Gly-His-Pro-Gly-Trp-Asn-Ala-Pro-Gly-Ala- .... (30 of 90)
	CM-2-I	Ala-Gly-Val-Lys-Asp-Tyr-Lys-Leu-Thr-Tyr-Tyr-Thr-Pro-Glu-Tyr-Glu-Thr-Lys-Asp-Thr-Asp-Phe-Leu-Ala-Ala-Phe-Arg-Val-Ser-Pro-Gln-Pro-Gly-Val- .... (34 of 125)
	CM-3	Pro-Gly-Val-Ile-Pro-Val-Ala-Ser-Gly-Gly-Ile-His-Val-Trp-Trp-His-Met (17)
Barley	CM-8	Ile-Lys-Gly-Ala-Val-Phe-Ala-Arg-Gln-Leu-Gly-Val-Pro-Glu-Lys-Asp-Gly-Ala ? -Ser-Ile-Thr-Phe ... (22 of 45)
Spinach		*Met-His-Arg*-Ala-Val-Phe-Ala-Arg-Gln .... (8 of 15)
Barley	CB-VI-A	Lys-Ala-Val-Ile-Asp-His-Arg-Gln .... (8 of 14)
Spinach		Lys Ala-Val-Ile-Asp-His-Arg-Gln .... (8 of 14)
Barley	CM-2-II	Met-Ser-Gly-Gly-Asp-His-Ile-His-Ser-Gly-Thr-Val-Val-Gly-Lys-Leu-Glu-Gly-Glu-Arg-Glu ..... (20 of 55)
Spinach		*Leu*-Ser-Gly-Gly-Asp-His-Ile-His-Ser-Gly-Thr-Val-Val-Gly-Lys-Leu-Glu-Gly-Glu-Arg

genome (plastome). On the basis of these incompatibilities, W. Stubbe has recognized five chloroplast genomes (I to V) which are adapted to particular nuclear genome classes (Kutzelnigg and Stubbe 1974). An isolation procedure for *Oenothera* ribulose bisphosphate carboxylase and a peptide mapping procedure to characterize the subunit polypeptides have been worked out (Holder 1976, 1978). The limited N-terminal sequencing of the small subunit of *Oenothera biennis* München and *parviflora ammophila* did not reveal any amino acid differences. Of primary interest is the question whether the five chloroplast genomes of *Oenothera* carry different allelic forms of the gene for the large subunit of ribulose bisphosphate carboxylase.

Isolated S-carboxymethylated large subunit was digested with chymotrypsin. The resultant soluble peptides were separated by ion exchange chromatography on a column of Dowex AG50W-X4 using a pyridine acetate gradient. Effluent fractions were analyzed by thin layer chromatography on silica gel, using isopropanol-acetic acid-water as solvent. Triplicate peptide maps were characterized by staining with cadmium ninhydrin reagent (peptides with N-terminal glycine are yellow, those with serine, threonine, asparagine or histidine are orange and others are red) and with four reagents specific for certain amino acid residues. Peptides containing arginine were located as a blue-green fluorescence with phenanthrenequinone, tyrosine peptides were identified as red spots using 1-nitroso-2-naphtol and nitric acid, tryptophan peptides were stained purple with Ehrlich's reagent and Pauly's reagent was used to localize histidine- and tyrosine-containing peptides as red and purple spots, respectively. The use of specific stain reactions greatly facilitated comparison of the peptide maps. For further resolution of complex regions in the peptide map, two dimensional thin layer chromatography of pooled fractions from the ion exchange column was carried out using four different solvent systems as desirable (isopropanol-acetic acid-water; amylalcohol-isopropanol-formic acid-water; butanol-methanol-acetic acid-water-pyridine; butanol-isopropanol-acetic acid-water-pyridine). Two peptides were only detected using chlorine/starch-KI which is sensitive for large peptides and independent of the presence of a free amino group. Altogether about 80 peptides were reliably mapped by this procedure.

A comparison of the large subunit chymotryptic peptide maps of nine *Oenothera* genotypes revealed a striking overall similarity in the patterns of the peptides, the number of clearly detectable differences being small. The presence or absence of four peptides designated A, B, C and D were found to vary in accordance with Table 3.

Some tentative conclusions can be drawn from these results. Since the majority of the chymotryptic peptides studied must be small, the peptide differences detected between the genotypes are likely to be the result of single

Table 3. Presence or absence of four chymotryptic peptides in the peptide maps of the large subunit of ribulose-1,5-bisphosphate carboxylase from nine *Oenothera* genotypes.

hookeri	Chloroplast	genome	I	$A^- B^- C^+ D^+$
strigosa	"	"	I	$A^- B^- C^- D^+$
elata Guatemala	"	"	I	$A^- B^+ C^+ D^+$
biennis München	"	"	II	$A^- B^+ C^+ D^+$
biennis Citronelle	"	"	III	$A^- B^+ C^+ D^+$
lamarkiana Schweden	"	"	III	$A^- B^- C^- D^+$
parviflora ammophila	"	"	IV	$A^+ B^+ C^+ D^-$
parviflora atrovirens	"	"	IV	$A^+ B^+ C^+ D^-$
argillicola	"	"	V	$A^+ B^- C^+ D^-$

amino acid replacements and indicate the evolutionary divergence of this chloroplast gene within the genus *Oenothera* in agreement with the expected polymorphism of its chloroplast DNA. According to Kutzelnigg and Stubbe (1974), the 'primitive' chloroplast genome IV is compatible with all nuclear complexes. It is found to be lacking peptide D in both representatives tested. Chloroplast genomes V and I are adapted to only a single nuclear genotype each. They are characterized by the absence of two or three peptides with the exception of *elata* which only lacks peptide A. Chloroplast genomes IV and V are considered to be related, which is also suggested by the fingerprint analysis, IV lacking peptide D and V lacking peptide D plus B, whilst peptide A is present in both. That the chloroplast genome classification, as defined by incompatibility relationships between the chloroplast and nuclear genomes, need not be reflected in the fingerprints of the large subunit is exemplified by the two representatives of chloroplast genome III, *biennis* Citronelle lacking peptide A and *lamarkiana* Schweden lacking peptides A, B and C. It is tempting to suggest that the differences detected in the chloroplast gene for the large subunit of ribulose bisphosphate carboxylase have arisen by single amino acid substitutions and recombination. The results encourage studies to define the alleles of this chloroplast gene in *Oenothera* at the nucleotide level and to investigate whether the large and small subunits transcribed and translated from different alleles in *Oenothera* are equally compatible to form a functional ribulose bisphosphate carboxylase/oxygenase oligomer.

The terminal amino acid sequence of the small subunit of ribulose bisphosphate carboxylase from the amphidiploid *Nicotiana tabacum* is polymorphic for isoleucine/tyrosine at position 7 and for asparagine/glycine at position 8 (Strøbæk, Gibbons, Haslett, Boulter and Wildman 1976). Examination of the equivalent positions in the parent species revealed isoleucine-asparagine in *Nicotiana sylvestris* and tyrosine-glycine in *N. tomentosiformis*, which allows the conclusion that the amphidiploid *Nicotiana tabacum* has inherited two alleles for the small subunit, one from each parent species. These alleles have been

retained for at least several hundred generations. The large subunit of the amphidiploid is in its isoelectric focusing pattern indistinguishable from the large subunit of *Nicotiana sylvestris* (Gray, Kung, Wildman and Sheen 1974) identifying the latter species as the female parent. The advantage of polyploids in a self-fertilizing species lies in the possibility to harbour two or more alleles at a gene locus to provide a greater versatility of gene action (von Wettstein 1943). Hetero-oligomeric enzyme molecules resulting from the presence of two different alleles at a locus may have selective advantages (Fincham 1972) and provide a better adaptability to the plant. In tobacco, two different homomeric forms and the seven heteromeric forms of ribulose bisphosphate carboxylase/oxygenase are possible in an octameric enzyme comprising the single large subunit type and the two different small subunit types. This offers an opportunity to study any physiological differences between these different molecular forms of the enzyme in tobacco.

Differences in banding patterns produced by isoelectric focusing are a reflection of the primary structure of the protein. This can be used in a first attempt to identify allelic differences between genes for the large subunit as well as between genes for the small subunit. The differences can be used to probe for the presence of the alleles in a cell culture or in a plant. Recently this technique permitted the identification of four somatic hybrids produced by fusion of protoplasts from tomato and dihaploid potato (Melchers, Sacristan and Holder 1978). The ribulose bisphosphate carboxylase enzyme oligomer in the four plants contained the small subunit products resulting from the expression of both tomato and potato nuclear genes. In three of the four plants, the large subunit polypeptides and hence the functional chloroplast DNA originated from tomato, whereas in the fourth the large subunit and therefore the chloroplast DNA was derived from potato. The prominence of the potato small subunit bands suggests that the latter near-hexaploid plant has probably arisen by the triple fusion of two potato protoplasts which had undifferentiated plastids and one tomato protoplast which had differentiated chloroplasts. The primary structure of the large subunit will be a useful marker to decide whether chloroplast genome competition was responsible for the dominating presence of potato chloroplast DNA in this hybrid.

Combinations of different allelic forms of the nuclear and chloroplast genes for ribulose bisphosphate carboxylase/oxygenase in a single cell and the formation of hybrid enzyme molecules in vivo or in vitro will facilitate analyses of conformational, functional and biosynthetic aspects of the enzyme.

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