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Expressed Genes for Plant-Type Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase in the Photosynthetic Bacterium *Chromatium vinosum*, Which Possesses Two Complete Sets of the Genes†

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Two sets of genes for the large and small subunits of ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) were detected in the photosynthetic purple sulfur bacterium *Chromatium vinosum* by hybridization analysis with RuBisCO gene probes, cloned by using the λ Fix vector, and designated *rbcL-rbcS* and *rbcA-rbcB*. *rbcL* and *rbcA* encode the large subunits, and *rbcS* and *rbcB* encode the small subunits. *rbcL-rbcS* was the same as that reported previously (A. M. Viale, H. Kobayashi, T. Takabe, and T. Akazawa, FEBS Lett. 192:283–288, 1985). A DNA fragment bearing *rbcA-rbcB* was subcloned in plasmid vectors and sequenced. We found that *rbcB* was located 177 base pairs downstream of the *rbcA* coding region, and both genes were preceded by plausible procaryotic ribosome-binding sites. *rbcA* and *rbcB* encoded polypeptides of 472 and 118 amino acids, respectively. Edman degradation analysis of the subunits of RuBisCO isolated from *C. vinosum* showed that *rbcA-rbcB* encoded the enzyme present in this bacterium. The large- and small-subunit polypeptides were posttranslationally processed to remove 2 and 1 amino acid residues from their N-termini, respectively. Among hetero-oligomeric RuBisCOs, the *C. vinosum* large subunit exhibited higher homology to that from cyanobacteria, eucaryotic algae, and higher plants (71.6 to 74.2%) than to that from the chemolithotrophic bacterium *Alcaligenes eutrophus* (56.6%). A similar situation has been observed for the *C. vinosum* small subunit, although the homology among small subunits from different organisms was lower than that among the large subunits.

Ribulose 1,5-biphosphate carboxylase/oxygenase (RuBisCO) is a central enzyme in CO_2 fixation in a wide range of autotrophic organisms, from photo- and chemoautotrophic procaryotes to higher plants. In plants, algae, and some autotrophic eubacteria, this enzyme is composed of octamers of each 50- to 55-kilodalton (kDa) large subunit (L, subunit A) and 12- to 18-kDa small subunit (S, subunit B), L_8S_8 (1, 3, 7, 60). The only known exceptions are RuBisCO from *Rhodospirillum rubrum* and form II RuBisCO from rhodopseudomonads, which are composed only of large subunits (1, 7, 60). Several amino acid residues in the active site have been identified as essential for activity by means of chemical modification or site-directed mutagenesis (7, 10, 20–22, 46). Despite intensive work, the function of the small subunit is not well understood, although it is necessary for the activation and catalysis of the hetero-oligomeric enzyme (1, 2, 7, 32, 60). The presence of two sets of genes coding RuBisCO has been reported for the rhodopseudomonads (60), *Alcaligenes eutrophus* (5, 28), and *Nitrobacter hamburgensis* (19). However, no sequence data for genes for L_8S_8-type RuBisCO from photosynthetic bacteria have been published.

Chromatium vinosum is a photosynthetic purple sulfur bacterium, recently assigned to the γ subdivision of purple photosynthetic bacteria and relatives, according to oligonucleotide signature analysis of 16S rRNA sequences (70, 71). Its RuBisCO has the L_8S_8 structure (1, 2) and kinetic properties intermediate between those of the enzymes from higher plants and from cyanobacteria (26, 27). This enzyme has been intensively studied as a model system for the formation of homologous and heterologous active hybrid molecules (2). It has been shown that the synthesis of large and small subunits in this bacterium is coordinated (31) and enhanced under photoautotrophic conditions in the presence of reduced sulfur compounds (30). This regulation occurs mainly at the level of transcription, both subunits being cotranscribed (66). Genes for the large and small subunits of RuBisCO from cyanobacteria (39, 53–55), eucaryotic algae (15, 17, 59), and higher plants (37, 40, 52) have been cloned and sequenced. In general, the amino acid sequences of large subunits are highly homologous, but those of small subunits are less conserved. We recently cloned one set of genes encoding the large and small subunits from *C. vinosum* and expressed them under the control of the *tac* promoter in *Escherichia coli* (67). We report here the presence of a second set of genes, *rbcA-rbcB*, for plant-type RuBisCO, which code for the subunits occurring in *C. vinosum*. We also present here the nucleotide sequence analysis of these genes and discuss aspects of the phylogeny of RuBisCO.

MATERIALS AND METHODS

DNA manipulations and sequencing. Isolation of bacterial plasmids or phage DNA, gene cloning, DNA digestion with restriction enzymes, labeling DNA with [α-^{32}P]dCTP, restriction mapping of end-labeled DNA fragments, and Southern blotting analysis were carried out following described

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conventional procedures (8). For total DNA isolation from *C. vinosum* D (either the strain maintained in our laboratory or the ATCC type strain 17899), autotrophic cultures were started from a single-cell isolate produced by the agar dilution procedure (44). A λ DNA library from the *C. vinosum* strain maintained in our laboratory was constructed by using the λ Fix vector (Stratagene), following the manufacturer's instructions. To obtain plasmids pCV17 and pCV23, in which a *C. vinosum* DNA sequence harboring the complete *rbcA-rbcB* gene is cloned in opposite orientations, DNA isolated from λ 53 (see Fig. 2) was completely digested with *Sal*I, ligated to *Sal*I-digested Bluescribe M13+ vector (Stratagene), and transformed into *E. coli* JM109 (72). The clones containing the *rbcA-rbcB* genes were detected by colony hybridization (8) with the ^{32}P-labeled 1.8-kilobase-pair (kbp) *Bam*HI DNA fragment containing most of *rbcL-rbcS* (67) as a probe. *Bam*HI and *Kpn*I restriction sites in pCV17 and pCV23 were employed to produce unidirectional deletions with exonucleases III and VII as described before (29). Deleted plasmids were ligated and transformed into *E. coli* JM101 (72).

Single-stranded DNA was produced essentially as described before (34), using either VCS-M13 (Stratagene) or R408-M13 (47) as a helper phage. The single-stranded DNA was sequenced by the chain-termination method (29, 48) with a modified T7 DNA polymerase (Sequenase; U.S. Biochemicals) and [α-^{35}S]dATP or [α-^{32}P]dCTP, as described by the manufacturer. Compression problems were resolved by replacing dGTP with dITP and sequencing the complementary strand from the series of plasmids containing deleted sequences. For sequencing some regions which were not appropriately covered by the obtained deletions, oligonucleotide primers (18-mers) were synthesized by the phosphoramidite method (9), with an Applied Biosystems 380A DNA synthesizer. Oligonucleotides were purified by a Mono Q anion-exchange column connected to a Pharmacia fast protein liquid chromatography (FPLC) system. A gradient of 0.5 to 0.65 M NaCl in 10 mM NaOH at a flow rate of 1 ml/min was employed. The purified oligonucleotides were used in place of universal primers. The software DNASIS (Hitachi) was used for the analysis of DNA, RNA, and protein sequences, including alignment of DNA and protein sequences, codon usage calculation, and predictions of RNA secondary structure and protein hydropathy. Final adjustments in protein sequence comparisons (see Fig. 5) were made by visual inspection.

RuBisCO purification, subunit isolation, and N-terminal sequence determination. RuBisCO was purified to homogeneity from photoautotrophically grown *C. vinosum* cells following described procedures (12). The large and small subunits were separated by means of a Superose 12 gel filtration column by FPLC. The separation was done with 50 mM sodium phosphate (pH 6.9)–1 mM EDTA–1% sodium dodecyl sulfate at a flow rate of 1 ml/min. The purified subunits were subjected to the manual Edman degradation procedure (11), and the phenylthiohydantoin-amino acids were determined by reversed-phase high-performance liquid chromatography (73).

Materials. All reagents were of analytical grade. Restriction and modification enzymes were obtained from Toyobo (Osaka) and Takara (Kyoto). Labeled nucleotides were from Amersham Corp. and New England Nuclear Corp.

RESULTS

Identification of two distinct DNA sequences containing RuBisCO genes. We had previously found one set of genes coding subunits of L_8S_8-type RuBisCO from *C. vinosum*, *rbcL-rbcS*, in a plasmid library constructed by using pUC8, and expressed them in *E. coli* under the control of the *tac* promoter (32, 67). However, the amino acid composition of the enzyme expressed in *E. coli* showed significant differences from RuBisCO isolated from *C. vinosum* (unpublished data). We thus decided to search for another set of genes encoding the enzyme present in *C. vinosum*. We prepared total DNA from *C. vinosum* which had been isolated as a single colony to confirm that there was no contamination with other organisms, digested it with different restriction enzymes, and carried out Southern blot analysis with the previously isolated *rbcL* and *rbcS* (67) as probes. As shown in Fig. 1, the positive signals in each digest (stars) could not be predicted from the restriction map which we had constructed for *C. vinosum rbcL-rbcS* (67). Exactly the same results were obtained with DNA isolated from either the ATCC type strain 17899 or the strain maintained in our laboratory.

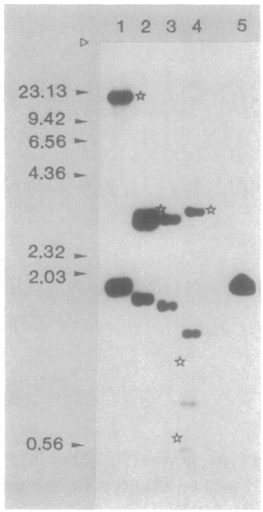

FIG. 1. Southern blot analysis of *C. vinosum* DNA with a RuBisCO gene probe. Total DNA from the bacterium was digested with several restriction enzymes and analyzed for the presence of genes for RuBisCO with *C. vinosum rbcL-rbcS* as a probe. Digests: lane 1, *Bam*HI; lane 2, *Bgl*I; lane 3, *Rsa*I; and lane 4, *Sal*I. Lane 5, 1.8-kbp *Bam*HI fragment containing most of *rbcL-rbcS* (67). The stars beside positive signals indicate bands which were not predicted from the physical map of previously isolated *rbcL-rbcS* (67). The positions of the *Hin*dIII-digested DNA size markers (in kilobase pairs) and the loading zone (open arrowhead) are indicated.

We thus made attempts to isolate the DNA fragment(s) producing signals indicated by the stars in Fig. 1. For this purpose, we constructed a λ library of total *C. vinosum* DNA and screened it with the *rbcL-rbcS* probe. Among 11 λ clones isolated, it was clear that two different kinds of DNA sequences harboring RuBisCO genes had been cloned. The physical maps of two selected λ clones, designated λ22 and λ53, are presented in Fig. 2. It was found that the cloned DNA fragments were derived from two different loci and that the hybridization signals observed in Fig. 1 were ascribable to the presence of two sets of genes for RuBisCO in *C. vinosum*. A DNA fragment cloned in λ22 produced the same restriction patterns as those observed in our previous work (67), indicating the presence of the *rbcL-rbcS* genes. In order to identify the putative RuBisCO genes cloned in λ53, we subcloned a *Sal*I fragment (Fig. 2) in the plasmid vector Bluescribe M13+, and selected two plasmids, pCV17 and

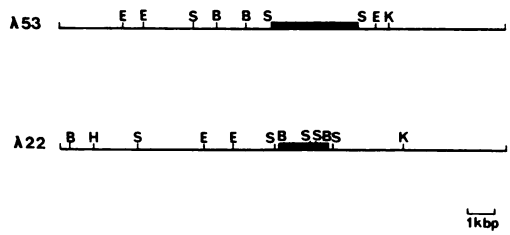

FIG. 2. Physical map of λ22 and λ53 clones and location of RuBisCO genes. The restriction enzyme maps were constructed as described in Materials and Methods. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I.

pCV23, containing the cloned *Sal*I fragment in opposite orientations. These plasmids were mapped with several restriction enzymes, and the positions of genes for the subunits were detected by the *rbcL* and *rbcS* probes (67). The locations of newly identified genes for large and small subunits, designated *rbcA* and *rbcB*, respectively, are shown in Fig. 3.

Sequencing strategy. The cloned DNA in plasmids pCV17 and pCV23 was digested unidirectionally with exonucleases III and VII in order to produce appropriate deletions for sequencing DNA. In this way, the sequence data for both DNA strands were obtained and overlapped to make unequivocal identification of the nucleotide sequence (Fig. 3). Some DNA regions which were not completely covered by the deletions were sequenced by replacing universal primers with synthesized oligonucleotides (Fig. 3).

Structures of *rbcA* and *rbcB*. The nucleotide sequence determined and deduced amino acid sequences for *rbcA* and *rbcB*, assuming no deviations from the universal genetic code, are given in Fig. 4. The fact that *rbcA* and *rbcB* code for the large and small RuBisCO subunits present in *C. vinosum* cells was established by the analysis of N-termini of subunits prepared from purified *C. vinosum* RuBisCO (see below). The coding sequences were separated by a 177-bp intergenic region and preceded by plausible ribosome-binding sites (Fig. 4). Downstream of the *rbcB* coding region, some palindromic sequences were present (Fig. 4). The sequences may form stem-and-loop structures in the transcript, serving as transcriptional termination signals. In

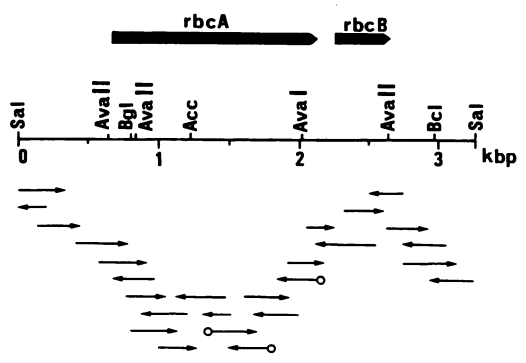

FIG. 3. Physical map and sequencing strategy for the *C. vinosum* *rbcA-rbcB* genes. Subclones for sequencing were made by digestion of plasmids pCV17 and pCV23 with exonucleases III and VII. The arrows indicate the extent and direction of sequences determined. Open circles show regions sequenced by use of synthesized oligonucleotide primers. The *rbcA* and *rbcB* coding regions, directions of the coding frames (5′ to 3′), and some restriction sites are presented. Abbreviations: Acc, *Acc*I; Bgl, *Bgl*I; Bcl, *Bcl*I; Sal, *Sal*I.

particular, the last stem-loop structure indicated in Fig. 4 is composed of a G+C-rich stem (5′-GCCCC-3′ and 5′-GGGGC-3′) and followed by a stretch of U residues in the transcript (5′-TTTT-3′ in DNA), features which are typical of rho-independent transcriptional termination signals in *E. coli* (45). An imperfect palindrome was also predicted in the intergenic region (Fig. 4), which may also form a hairpin structure. A similar situation has been described for the intergenic region of *rbcL* and *rbcS* from the cyanobacterium *A. nidulans* (54). The function of this structure is unknown, although it may be related to premature termination of transcription during an excess of gene product. The promoter region of *rbcA-rbcB* has not been experimentally identified yet, and no consensus sequences for *E. coli* promoters could be found.

The *C. vinosum rbcA* and *rbcB* coding regions had average G+C contents of 64 and 60%, respectively, which are close to 61.3 to 66.3% reported for the total DNA in this organism (65). The codon usages of *rbcA* and *rbcB* are shown in Table 1. Codon usages in the two genes were similar, with the interesting exception of the UCG (Ser) codon, which was not found in *rbcB* but found frequently in *rbcA*. As expected for organisms with high G+C contents, a clear bias toward the use of G and C, especially at the third position in the codon, was observed. It is of interest that the codon usage in *C. vinosum rbcA* is close to that found in *R. rubrum* and *A. eutrophus* (4) and shows greater differences from codon usage in cyanobacteria and eucaryotic organisms (4).

N-terminal analysis of the large and small subunits. RuBisCO was purified to homogeneity from autotrophically grown *C. vinosum* cells, and the subunits were separated by gel filtration FPLC in the presence of sodium dodecyl sulfate. The purified polypeptides were subjected to manual Edman degradation. For the large subunit, the sequence predicted from the DNA data was Met-Ala-Lys-Thr-Tyr-Ser-Ala-Gly-Val (Fig. 4), and the chemically obtained amino acid sequence was Lys-Thr-Tyr-Ser-Ala-Gly-Val. For the small subunit, the deduced sequence was Met-Ser-Glu-Met-Gln-Asp-Tyr-Ser, and the chemically obtained amino acid sequence was Ser-Glu-Met-Gln-Asp-Tyr-Ser. The results obtained from the Edman degradation analysis of the N-terminal regions of both polypeptides are thus completely consistent with the deduced amino acid sequences and also allowed the identification of *rbcA* and *rbcB* as the coding sequences for RuBisCO expressed in *C. vinosum*. The N-terminal sequences of subunits synthesized by the direction of *rbcL-rbcS* under the control of the *tac* promoter in *E. coli* differ substantially from the above-described sequences (unpublished data). It can also be observed that both subunits were processed after translation in *C. vinosum* cells. Two amino acid residues have been removed from the N terminus of the large subunit, and only the formylmethionine residue has been taken out from the small subunit.

These N-terminal sequences are in agreement with those determined by Edman degradation by Torres-Ruiz and McFadden (64) with the following exceptions. For the large subunit, they reported that the sequence started with Ser-Lys-Thr-Tyr-Ser. We could not find Ser as the N-terminal residue, and we obtained a sequence initiating with Lys-Thr-Thy-Ser as described above. We deduced Ala instead of the Ser at one residue before the Lys from the DNA sequence (Fig. 4), and indeed the presence of the Ala has been confirmed by Edman degradation of the N-terminal region of the large subunit produced in *E. coli* cells (unpublished data). Another difference appears at position 17 in the large subunit (Fig. 4), at which we deduced Trp instead of

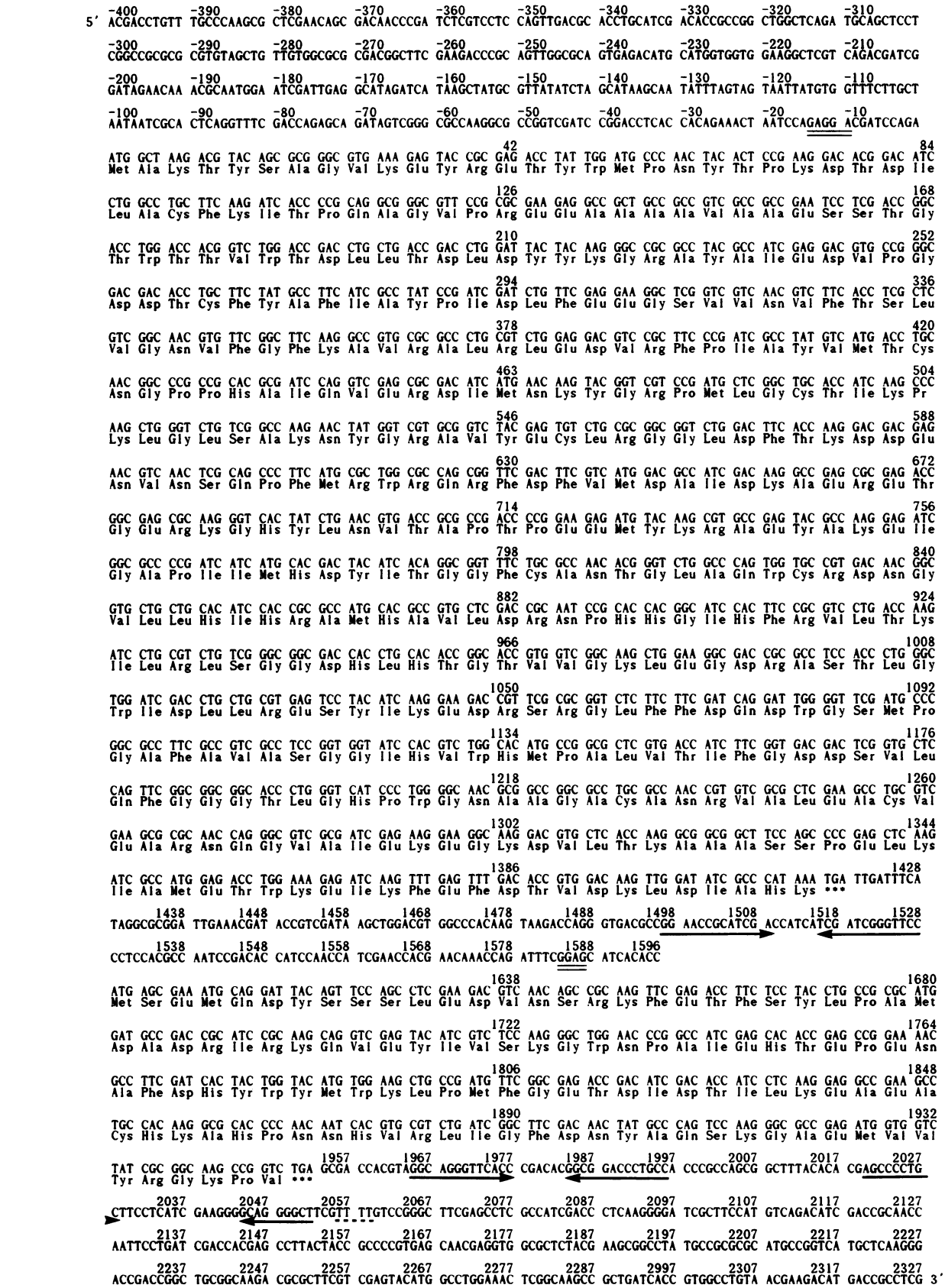

FIG. 4. Nucleotide sequence of *C. vinosum rbcA-rbcB* and deduced amino acid sequences. The upper large reading frame (1 to 1419) is for *rbcA*, and the lower short one (1600 to 1956) is for *rbcB*. Putative ribosome-binding sites preceding both coding frames are underlined with double lines. The arrows indicate palindromic sequences. The dotted line corresponds to a stretch of U residues at a possible terminator of the transcript. See the text for details.

TABLE 1. Comparison of codon usage in *rbcA* and *rbcB*

Amino acid	Codon	% in each amino acid (actual no.)	
		rbcA	*rbcB*
Phe	UUU	10 (2)	0 (0)
	UUC	90 (19)	100 (5)
Leu	UUA	0 (0)	0 (0)
	UUG	3 (1)	0 (0)
	CUU	0 (0)	0 (0)
	CUC	27 (9)	40 (2)
	CUA	0 (0)	0 (0)
	CUG	70 (24)	60 (3)
Ile	AUU	0 (0)	0 (0)
	AUC	100 (25)	100 (6)
	AUA	0 (0)	0 (0)
Met	AUG	100 (13)	100 (6)
Val	GUU	3 (1)	0 (0)
	GUC	59 (19)	71 (5)
	GUA	0 (0)	0 (0)
	GUG	38 (12)	29 (2)
Tyr	UAU	33 (6)	29 (2)
	UAC	77 (12)	71 (5)
Ser	UCU	0 (0)	0 (0)
	UCC	31 (5)	50 (4)
	UCA	0 (0)	0 (0)
	UCG	56 (9)	0 (0)
	AGU	0 (0)	12 (1)
	AGC	13 (2)	38 (3)
Pro	CCU	0 (0)	0 (0)
	CCC	30 (6)	17 (1)
	CCA	0 (0)	0 (0)
	CCG	70 (14)	83 (5)
Thr	ACU	3 (1)	0 (0)
	ACC	80 (24)	100 (4)
	ACA	3 (1)	0 (0)
	ACG	14 (4)	0 (0)
Ala	GCU	6 (3)	0 (0)
	GCC	67 (32)	78 (7)
	GCA	0 (0)	0 (0)
	GCG	27 (13)	22 (2)
His	CAU	13 (2)	0 (0)
	CAC	87 (13)	100 (5)
Gln	CAA	0 (0)	0 (0)
	CAG	100 (8)	100 (3)
Asn	AAU	7 (1)	17 (1)
	AAC	93 (14)	83 (5)
Lys	AAA	12 (3)	0 (0)
	AAG	88 (23)	100 (8)
Asp	GAU	16 (5)	38 (3)
	GAC	84 (27)	62 (5)
Glu	GAA	30 (9)	36 (4)
	GAG	70 (21)	64 (7)
Cys	UGU	11 (1)	0 (0)
	UGC	89 (8)	100 (1)
Trp	UGG	100 (10)	100 (3)
Arg	CGU	33 (9)	20 (1)
	CGC	63 (17)	80 (4)
	CGA	0 (0)	0 (0)
	CGG	4 (1)	0 (0)
	AGA	0 (0)	0 (0)
	AGG	0 (0)	0 (0)
Gly	GGU	30 (13)	0 (0)
	GGC	70 (30)	100 (5)
	GGA	0 (0)	0 (0)
	GGG	0 (0)	0 (0)

Tyr from the DNA sequence. In the small subunit, the amino acid residues at positions 5, 10, and 30 differ in our data and theirs (64). At these positions, we obtained Gln, Ser, and Ala residues, respectively (Fig. 4). We also deduced Asp at position 31, which was not found before (64). Although Torres-Ruiz and McFadden (64) described their strain as *C. vinosum* D, we may conclude that they used another strain of *C. vinosum* than the ATCC type strain 17899 which we analyzed.

Structure comparison of large subunits. *C. vinosum rbcA* encodes a polypeptide of 472 amino acids with a molecular weight of 52,521, in the 50- to 55-kDa range reported for large subunits from other organisms (1, 4, 7). As shown in Table 2, *rbcA* exhibited higher homology to its counterparts from cyanobacteria, algae, and higher plants than to those from the purple bacterial group so far sequenced. At the nucleotide level, homology ranged between 63.0% (to tobacco) and 73.9% (to cyanobacteria), whereas the homology to the *R. rubrum* large subunit was as low as 51.2%. At the polypeptide level, higher homologies were observed to large subunits from cyanobacteria, eucaryotic algae, and higher plants (71.6 to 74.2%) than to the large subunit from the chemoautotrophic bacterium *A. eutrophus* (56.6%), although very low homology (26%) was found to the *R. rubrum* large subunit. Most of the differences in homology at the polypeptide level can be attributed to conservative amino acid substitutions (Fig. 5). When identical and equivalent amino acid sequences were compared by a mutation data matrix to estimate related residues (51), the homology between the large subunits from *C. vinosum* and other species increased,

TABLE 2. Sequence homologies between *C. vinosum rbcA* and genes for large subunits from other organisms

Species[a]	% Sequence homology[b]		
	Nucleotides	Amino acids	
		Identical	Related[c]
A. nidulans	73.9	74.2	86.7
C. reinhardtii	64.3	73.1	84.1
N. tabacum	63.0	71.6	82.4
A. eutrophus	66.3	56.6	73.7
R. rubrum	51.2	26.0	41.5

[a] Species are listed in order of highest to lowest amino acid homology.
[b] To calculate the homologies, nucleotides or amino acids at identical positions in the sequences were compared after maximum matching. The alignments shown in Fig. 5 were used for the calculation.
[c] Related residues are counted in considering scores higher than 0.12 in the mutation matrix MDM_{78} of Schwartz and Dayhoff (51).

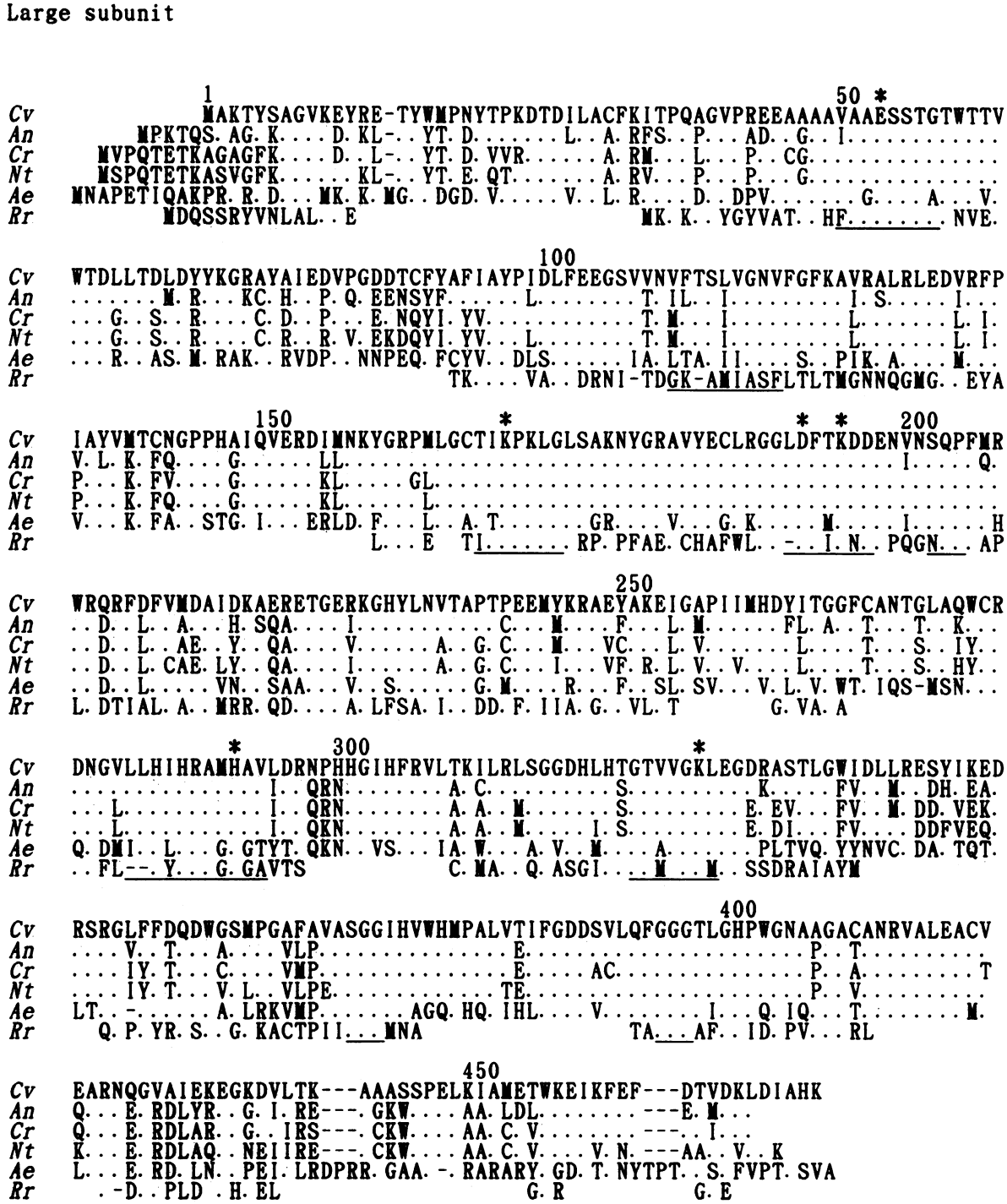

FIG. 5. Comparison of the polypeptide sequences of the large and small subunits of RuBisCO from *C. vinosum* (*Cv*) and other organisms. Published DNA sequence data were taken from the data bases of GenBank (Release 52.0) and EMBL (Release 13.0) or directly input to a computer and converted to protein sequences. Large and small subunits from the cyanobacterium *A. nidulans* (*An*) (53, 55), the eucaryotic algae *Cyanophora paradoxa* (*Cp*) (59) and *Chlamydomonas reinhardtii* (*Cr*) (15, 17), the higher plant *Nicotiana tabacum* (*Nt*) (37, 52), the chemolithotrophic bacterium *A. eutrophus* (*Ae*) (4), and the photosynthetic bacterium *R. rubrum* (*Rr*) (38) are shown. For the *R. rubrum* large subunit, only regions homologous to the *C. vinosum* large subunit are presented. For *C. reinhardtii* and *N. tabacum*, the transit sequences of the small subunit are not shown. The species are ordered from highest to lowest amino acid homology (see Tables 2 and 3). Residue numbers correspond to those in the *C. vinosum* sequences. Dots indicate the presence of the same residue at the position. Insertions are indicated by dashes. Asterisks indicate conserved residues which have been reported to be essential for enzyme activity. The sequences which have been revealed to be parts of the catalytic site in the tobacco enzyme are underlined. For details, see the text.

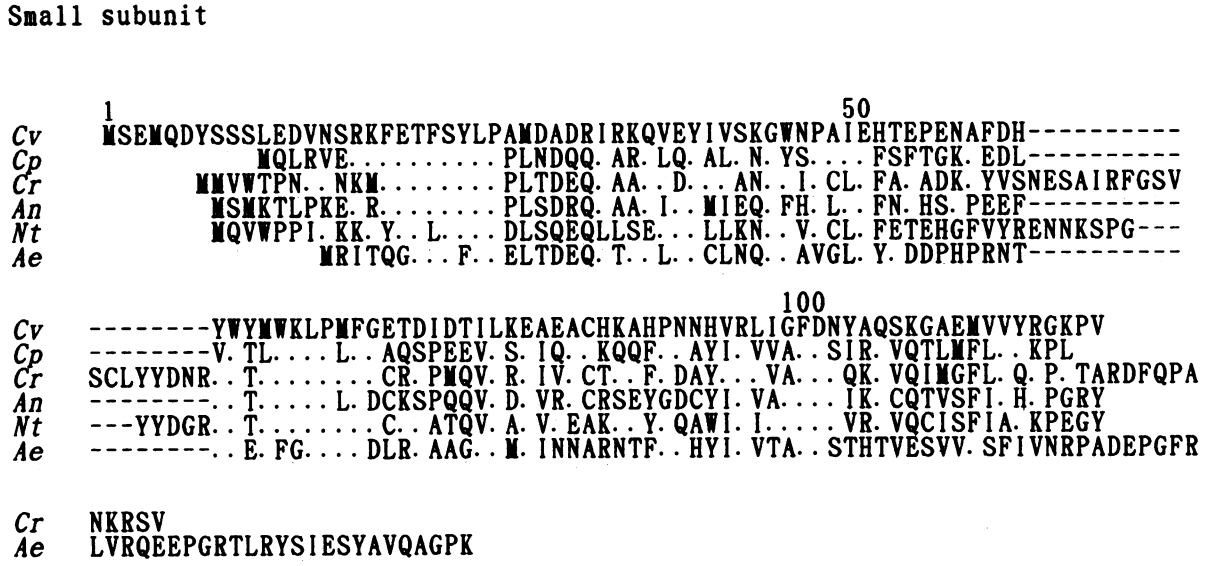

FIG. 5—*Continued*

ranging from 73.7% (to *A. eutrophus*) to 86.7% (to cyanobacteria). In the case of *R. rubrum*, an increase in homology was also observed, reaching 41.5% (Table 2).

In the *C. vinosum* large subunit, Lys-193 corresponds to the Lys residue involved in carbamylation and activation of the enzyme (36). The sequence enclosing this residue is of particular interest, since it was completely identical, from residues 162 to 207, to those of the RuBisCOs found in eucaryotic algae and higher plants (Fig. 5). The N-terminal regions of the large subunits were very variable in homology and length; the *C. vinosum* large subunit was the shortest and that of *A. eutrophus* was the longest (Fig. 5), suggesting that this region is not essential for enzyme functions. Variability in the C-terminal region of large subunits was not so marked. However, it is of interest that several insertions occurred in the C-terminal region of the *A. eutrophus* large subunit which were not present in large subunits from *C. vinosum* and other species (Fig. 5).

Hydropathy plots of the large subunits from *C. vinosum* RuBisCO and the other hetero-oligomeric enzymes, following a procedure described by Kyte and Doolittle (35), revealed similar distributions of hydrophilic and hydrophobic regions along the polypeptide (data not shown). These results indicate the common distribution of external and internal portions in the folded polypeptide chain in L_8S_8-type RuBisCOs.

Structure comparison of small subunits. *C. vinosum rbcB* encodes a polypeptide of 118 amino acids with a molecular weight of 13,794, a size generally reported for small subunits from other species (1, 7, 60). Much lower homology than that observed among the large subunits was found for the small subunits (Table 3). At the nucleotide level, homologies ranged between 51.5 and 54.5%. At the polypeptide level, however, there were unexpectedly lower homologies, ranging from 28.8% (to *A. eutrophus*) to 43.2% (to *Chlamydomonas reinhardtii*). It is also notable that the homologies were higher to small subunits from cyanobacteria, algae, and *Cyanophora paradoxa* than to the small subunit from *A. eutrophus*. These numbers somehow increased when related amino acids were accounted for (Table 3).

A comparison of amino acid sequences is presented in Fig. 5. The N- and C-terminal regions of the small subunits show great variability in sequence and length. In particular, the *C. vinosum* small subunit had a longer N-terminal sequence than the other mature polypeptides, although some conserved regions existed in the small subunit. Higher plants and *C. reinhardtii* contained insertions of 12 and 18 amino acids, respectively, in the middles of the peptides (Fig. 5). This kind of insertion was not present in the small subunits from *C. vinosum* and other procaryotes, as well as *C. paradoxa*.

DISCUSSION

Structure and function of the large subunit. The Lys-193 in *C. vinosum* RuBisCO corresponds to the Lys residue that undergoes carbamylation during enzyme activation (Lys-201 and Lys-191 in plant and *R. rubrum* large subunits, respectively) (7, 36) and is located in a highly conserved region (Fig. 5). Most of other amino acid residues identified as essential by site-directed mutagenesis, affinity labeling, or chemical modification are conserved in *C. vinosum*, such as Glu-52 (20), Cys-164 (49), Lys-167 and Lys-326 (21, 22, 49), Asp-190 (18), and His-290 (24, 41) (residues are numbered according to their positions in the *C. vinosum* large subunit). The Cys-164, close to the active-site Lys-167, has been modified by affinity reagents in the spinach enzyme (49). This Cys is conserved in the large subunit of *C. vinosum* but not in the subunits from *A. eutrophus* (4) and *R. rubrum* (38),

TABLE 3. Sequence homologies between *C. vinosum rbcB* and genes for small subunits from other organisms[a]

Species	% Sequence homology		
	Nucleotides	Amino acids	
		Identical	Related
C. paradoxa	53.3	38.1	58.5
C. reinhardtii	51.5	43.2	57.6
A. nidulans	54.3	38.1	56.8
N. tabacum	55.4	35.6	50.8
A. eutrophus	54.5	28.8	46.6

[a] See Table 2 footnotes.

and the role of the Cys is thus uncertain. We found that Met-451 is present in the *C. vinosum* large subunit at the position of a Cys residue known to be modified by affinity labels in the spinach enzyme (22). As shown in Fig. 5, this position is also occupied by different residues in *A. nidulans*, *A. eutrophus*, and *R. rubrum*, obscuring the role of the Cys as an essential residue. In the *R. rubrum* large subunit, His-44 and Cys-58 are labeled by affinity reagents (23) and Met-330 was subjected to site-directed mutagenesis (63). Neither of these residues are conserved in the *C. vinosum* RuBisCO and other hetero-oligomeric enzymes (Fig. 5), but the surrounding amino acid sequences are relatively common.

Structure and function of the small subunit. The amino acid sequences of small subunits have not been conserved as in large subunits (Fig. 5). However, three conserved regions can be seen in the small-subunit polypeptide sequences. One of them is found close to the N-terminal region, from positions 19 to 26. Another relatively conserved region is present between residues 61 and 70. The third one, close to the C-terminus, showing lower homology but conservative substitutions, spans residues 94 to 100. At least one of these regions seems to interact with the large subunit in tobacco (14). Some highly conserved residues can also be observed: Tyr-39, Gly-44, Glu-50, Glu-81, Leu-79, Arg-95, and Asp-100 (Fig. 5). Further studies are needed to elucidate the role of these conserved regions, as well as the functions of small subunits in hetero-oligomeric RuBisCOs.

Expression of the previously reported genes *rbcL* and *rbcS*. Deduced amino acid sequences from the DNA sequence of *rbcL-rbcS* (unpublished) are different at several residues from those of the large and small subunits of RuBisCO purified from *C. vinosum*, whereas the *rbcA-rbcB* nucleotide sequence (Fig. 4) let us provide the same N-terminal amino acid sequences as those of the subunits of *C. vinosum* RuBisCO. Our results thus show that *rbcA* and *rbcB* products are much more abundant than those of *rbcL* and *rbcS* in *C. vinosum*. In our preceding publication (66), we determined transcript levels for RuBisCO by hybridization with DNA fragments containing *rbcL*, *rbcS*, or both. Since *rbcL* and *rbcA* hybridize to each other even under stringent conditions (Fig. 1), we realized that we could potentially detect transcripts for both sets of genes, *rbcL-rbcS* and *rbcA-rbcB*, in the hybridization experiment (66). In order to determine the level of individual transcript from each of the genes, gene-specific DNA probes complementary to 3′ or 5′ untranslated regions must be used in hybridization experiments. At this stage, we raise the following possibilities: (i) *rbcL-rbcS* is less transcribed than *rbcA-rbcB*; (ii) *rbcL-rbcS* is transcribed as highly as *rbcA-rbcB* but transcripts for *rbcL-rbcS* are not efficiently translated; and (iii) peptides encoded by *rbcL-rbcS* are rapidly degraded posttranslationally.

Duplication of genes for RuBisCO in *C. vinosum*. We have revealed the presence of two complete sets of genes for L_8S_8-type RuBisCO in the photosynthetic purple bacterium *C. vinosum* in this study. Among the 11 λ clones analyzed, 5 were classified as carriers of *rbcL-rbcS* and 6 as carriers of *rbcA-rbcB*. We did not detect any clone of the locus for the large subunit alone without that for the small subunit. The presence of RuBisCO composed only of large subunits has been reported for *C. vinosum* by Torres-Ruiz and McFadden (64). However, as these authors concluded, it was a dissociated component during isolation (64) rather than the product of an independent gene for the large subunit. The results reported here, consistent with the data of hybridization experiments reported by others (56), seem to exclude the presence of a gene coding only for the large subunit in *C. vinosum*, such as that for *R. rubrum* RuBisCO or form II RuBisCO of the rhodopseudomonads (7, 60).

C. vinosum belongs to the γ subgroup of the purple photosynthetic bacteria and relatives, according to oligonucleotide signature analysis (70, 71). In these bacteria, only two RuBisCO genes have so far been sequenced: a gene for an L_2-type enzyme from *R. rubrum* (38) (α subgroup; 70, 71) and chromosomally encoded genes for L_8S_8-type RuBisCO from *A. eutrophus* (4, 6) (β subgroup). Interestingly, when amino acid sequences are compared, it is seen that *C. vinosum* RuBisCO is closer in homology to the cyanobacterial and higher-plant counterparts than to the enzyme from *A. eutrophus*, which seems to have accepted a larger number of mutations (Tables 2 and 3 and Fig. 5). However, the conserved sequences in all large and small subunits (although more restricted in the latter) strongly suggest a common origin for all RuBisCOs. For *C. vinosum*, it seems appropriate to exclude the possibility of lateral gene transfer of one of genes for the large and small subunits, considering the close G+C content values for both subunits and the similar codon usages (Table 1).

Two distinct sets of genes for RuBisCO (similar to phosphoribulokinase) have been described for the rhodopseudomonads (7, 60), as well as for the chemolithotrophs *A. eutrophus* (5, 6, 28) and *N. hamburgensis* (19). We have now found that it is also the situation in *C. vinosum*, in which two complete sets of genes for L_8S_8-type RuBisCO are present. These observations suggest that duplication of RuBisCO genes, properly of the carbon fixation (*cfx*) gene cluster containing genes at least for RuBisCO and phosphoribulokinase, was not an impossible event during evolution of the purple photosynthetic bacterial group. Actually, gene duplication has been observed in a variety of loci in several procaryotes (58, 68). These gene duplications can be selected by the need for an increased concentration of a gene product (58, 68), a state that is not difficult to imagine for RuBisCO, considering its poor efficiency as a catalyst (7). Thus, it is likely that duplication of genes for the large and small subunits has taken place during evolution of the purple photosynthetic bacteria and that the copies evolved at different rates. One of them may have evolved in such a way as to lose partially or completely the small subunit, as in the rhodopseudomonads or *R. rubrum* (60). In this context, it has already been noted that there is low homology between the C-terminal regions of L_2-type *R. rubrum* RuBisCO and a conserved region of small subunits (7); the homology is slightly higher to the *C. vinosum* small subunit. In this scheme, *R. rubrum* may have lost not only the small subunit, but also one region containing genes for L_8S_8-type RuBisCO. It has already been observed in *E. coli*, also a member of the purple bacteria (70, 71), that duplicated segments can be lost by homologous *recA*- or *red*-promoted recombination within the duplicated regions (68). Some answer will be obtained when DNA sequences for RuBisCOs from the rhodopseudomonads and other bacteria possessing two sets of *cfx* genes are completely elucidated.

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