Truncation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) from *Rhodospirillum rubrum* affects the holoenzyme assembly and activity

Benoit Ranty, Tomas Lundqvist¹, Gunter Schneider¹, Mike Madden², Richard Howard² and George Lorimer²

Department of Plant Physiology, URA CNRS No. 241, Paul Sabatier University, 118, route de Narbonne, F-31062 Toulouse Cedex, France, ¹Department of Molecular Biology, Swedish University of Agricultural Sciences, Biomedical Center, Box 590, S-75124 Uppsala, Sweden and ²Central Research and Development Department, E.I. DuPont de Nemours and Co., Wilmington, DE 19898, USA

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Truncations of the subunit of ribulose biphosphate carboxylase/oxygenase (Rubisco) from *Rhodospirillum rubrum* were generated by site-directed mutagenesis to examine the role of the C-terminal tail section. Removal of the last and the penultimate α -helices in the tail section changes the quaternary structure of the protein. Electrophoretic and electron microscope analysis revealed that the truncated subunits assemble into an octamer, whereas the wild-type enzyme has a dimeric structure. The octomerization of the mutant protein is due to a hydrophobic patch exposed to the solvent by truncation of the subunit. The mutant protein thus consists of four dimers, bound end-to-end by hydrophobic interactions. Insertion of a polar amino acid in the hydrophobic patch by a L424 to N424 substitution restores the familiar dimeric structure. Truncation of the subunit is associated with a considerable decrease in catalytic activity. The mutants undergo carbamylation but bind the reaction intermediate analog, 2-carboxy arabinitol-1,5-bisphosphate, poorly. This indicates that loss of activity in the mutant is due to weakened substrate binding. These findings suggest that the mutations in the tail section of the subunit are transmitted to the active site, although the C-terminal region is far from the active site. On the basis of the crystal structure of Rubisco, we propose a model for how the truncations of the enzyme subunit induce conformational changes in one of the two phosphate binding sites.

Key words: Rubisco/site-directed mutagenesis/hydrophobic interactions/phosphate binding site

Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is a key enzyme in photosynthetic and photorespiratory carbon metabolism. The enzyme catalyzes the carboxylation or the oxygenation of ribulose-1,5-bisphosphate (RuBP), the initial steps of the Calvin cycle and photorespiration, respectively (Lorimer, 1981a).

Rubisco from most photosynthetic organisms is a hexadecameric complex consisting of eight large (L, 50–55 kD) and eight small (S, 12–8 kD) subunits. In some photosynthetic bacteria another form of the protein in which the

small subunits are absent has been identified. The simplest type, a dimer of L, is present in *Rhodospirillum rubrum* (Tabita and McFadden, 1974). Apart from their subunit composition, these two forms of Rubisco differ by their primary structure. There is a low homology between the large subunits of the L₂ and L₈S₈ Rubiscos (Nargang *et al.*, 1984). However, some peptide regions are highly conserved, and residues identified as reactive groups of the active site are located in these homologous sequences (Andrews and Lorimer, 1987). Other similarities between the two forms of Rubisco have emerged from crystallographic studies. The structures of *R. rubrum* L₂ (Schneider *et al.*, 1986), tobacco L₈S₈ (Chapman *et al.*, 1987, 1988) and spinach L₈S₈ (Andersson *et al.*, 1989; Knight *et al.*, 1989) have been solved and the three-dimensional models show close relationships. In short, the L₈S₈ type can be described as four dimers similar to *R. rubrum* L₂, combined with two tetramers of S located at the poles of the L₈ core. Each large subunit is composed of a short N-terminal and a large C-terminal domain which has a β/α barrel structure. At the C-terminus, the polypeptide chain forms a tail extension which sits on a side of the barrel (Figure 1). The β/α barrel, first described in triose phosphate isomerase (Banner *et al.*, 1975), has been found to occur in several genetically and functionally non-related proteins. This folding pattern thus appears as a stable structural motif from which different biological functions can be designed (Lasters *et al.*, 1988).

In Rubisco, as in other proteins containing a β/α barrel, the active site is found on one side of the barrel at the carboxy end of the β -strands. Probing the active site of Rubisco by chemical and genetic experiments has identified some amino acids essential for catalysis (Lorimer, 1981b; Estelle *et al.*, 1985; Hartman *et al.*, 1987; Lorimer and Hartman, 1988). Lys191 of *R. rubrum* Rubisco is the site of carbamylation during the activation process, and Lys166 is thought to catalyze the enolization of RuBP, the initial step in the enzymatic reaction. Crystallographic analysis of Rubisco from *R. rubrum* (Schneider *et al.*, 1986), spinach (Andersson *et al.*, 1989) and tobacco (Chapman *et al.*, 1987) has shown that the active site of the enzyme is found at the interface between subunits. Residues from the N-terminal domain of one subunit and the C-terminal domain from the second subunit built up one active site. Such an arrangement requires a dimer as the minimal functional unit of the enzyme. Elegant site-directed mutagenesis experiments by Hartman and co-workers also reveal that the active site is located between subunits (Larimer *et al.*, 1987).

Structure–function relationships in the large subunit have mostly been focused on those regions considered as parts of the active site. In this paper, we have investigated the role of the tail section of *R. rubrum* Rubisco. This section accounts for the last 50 amino acid residues of the large subunit, and forms a tail extension beyond the barrel structure. In the *R. rubrum* enzyme, this peptide region is composed of four α -helices while only two are present in

Fig. 1. Schematic picture of the subunit of Rubisco from *R. rubrum*. Helices are shown as cylinders and β -strands as arrows. The nomenclature of the secondary structural elements is included. The small arrows indicate the points at which the polypeptide chain was truncated in the various mutants.

Fig. 2. Comparison of the amino acid sequences in the C-terminal tail section of Rubisco large subunit from spinach, *R. rubrum* and of a conserved region of the small subunit. The α helices are designated as α G, α H, α I and α J in *R. rubrum*, and α C1 and α C2 in spinach. A dash indicates a deletion inserted to maximize the structural alignment of these sequences. Amino acids in boxes show the residues at which mutations were introduced to produce deletion mutants of *R. rubrum* Rubisco.

the spinach subunit (Figure 2). Furthermore, a short sequence (residues 456–466) in *R. rubrum* shows some homology with a highly conserved region of the small subunit (residues 10–20). In order to assess the role of these elements, deletions from the tail section have been performed by site-directed mutagenesis. The truncated proteins obtained by expression of the mutant genes in *Escherichia coli* were analyzed with respect to the subunit composition and catalytic activity. The results demonstrate that truncations of the tail section of the large subunit significantly modify the subunit assembly and the enzymatic activity.

Results

Structural integrity of mutant proteins

The strategy for the construction of the truncated mutant gene combined oligonucleotide-directed mutagenesis with the cassette method. A major advantage of this procedure avoids the concern about the inadvertent introduction of secondary mutations outside the region of the gene under study. Truncations at positions E458, P449 or P441 were produced by changing the codons for these residues into stop codons. To confirm that the truncations were located at the C-termini of the mutant proteins, the 3' ends of the mutant genes were sequenced. The inserted stop codons were found in the expected positions. Restriction analyses of the 5' ends of the mutant genes were also performed, looking specifically for

the presence of the *Ava*II cleavage site which immediately follows and includes the start codon ATG. This *Ava*II site was detected in all mutant genes. It is therefore concluded that the N-termini of the truncated proteins are unaltered and that the truncations occurred at the C-termini, as expected.

The truncated mutant carboxylases obtained by expression of the different constructs in *E. coli* were first identified by non-denaturing electrophoresis of cell lysates (Figure 3). Immunodetection showed that Rubisco from mutant E458 (lane 2) co-migrated with the wild-type enzyme (lane 1), thus indicating a dimeric structure. Surprisingly, the protein extract from mutant P449 generated two immunoreactive bands (lane 3). One band migrated faster than the wild-type enzyme. However, this difference in electrophoretic mobility was consistent with the predicted alteration of charge and/or size of the mutant Rubisco. The other band exhibited a slower mobility, with an increased apparent molecular weight. This latter form became predominant in the protein extract from mutant P441 (lane 4).

Mutant forms of Rubisco were purified to apparent homogeneity, as judged by polyacrylamide gel electrophoresis (Figure 4). In the presence of SDS, the truncated subunit showed the expected slight decrease in molecular weight, relative to the wild-type subunit ($M_r = 50.5$ kd). As an example, the relative molecular weight of 48 kd evaluated from the mutant P441 subunit is in agreement with the reduction of size deduced from the amino acid sequence.

Fig. 3. Immunodetection of Rubisco from cell lysates of *E. coli* after electrophoresis in non-denaturing conditions on a 7.5% polyacrylamide gel. *E. coli* cells harbor wild-type (lane 1) or mutant Rubiscos (lanes 2, 3 and 4) excised at positions 458, 449 and 441, respectively.

More interestingly, electrophoretic patterns in denaturing conditions from mutants P449 and P441 revealed only one band identified as the large subunit (Figure 4B, lanes 3 and 4). This indicated that the high molecular form of Rubisco from these mutants is a polymer of large subunits. The molecular weight of the native complex was estimated to be ~440 kD by polyacrylamide gradient gel electrophoresis (Figure 4A). This data is consistent with an L_8 form of the mutant Rubisco. However, molecular weight determination by electrophoresis does not permit an accurate assessment of the subunit composition of the mutant protein. Therefore, electron microscope observations of negatively stained samples of Rubisco were performed.

Purified carboxylase from mutant P441 contained a homogeneous population of particles, exhibiting some resemblance to L_8S_8 Rubisco (Figure 5). The mutant protein had a ring-like structure measuring 11.8 ± 0.9 nm in diameter. The negative staining revealed a large central dark area that appears as a striking difference from the L_8S_8 structure. The L_8S_8 sample from *Anacystis nidulans* showed a four-lobed structure (~10.8 nm in diameter) surrounding a small central dot. The substructure of the mutant protein was not directly visible because of poor resolution in the periphery of the particle, but the resolution could be enhanced by rotational analysis. The 4-fold and 8-fold rotations clearly showed a symmetry. The best image was achieved with four rotation steps, thus suggesting a 4-fold symmetry. All these data reveal common features with the structure of L_8S_8 or the L_8 core from Rubisco partially depleted in small subunits (Andrews *et al.*, 1981). In combination with the molecular weight of the native protein, the structural observations are consistent with the mutant protein being composed of eight large subunits.

Subunit interactions in the mutant protein

To assess the basis of subunit assembly in the L_8 mutant, truncations of the large subunit have been modelled from

Fig. 4. Electrophoresis of purified Rubisco in the absence (A) or presence (B) of SDS. Lanes 1 and 5: wild-type Rubisco; Lanes 2, 3 and 4: mutant protein excised at position 458, 449 or 441, respectively. Lane 6: molecular weight markers. A polyacrylamide gradient gel (5–15%) and a homogeneous gel (10% in acrylamide) were used for non-denaturing and SDS electrophoresis, respectively. Proteins were stained in 0.1% Coomassie blue.

the coordinates of *R. rubrum*, and modifications at the surface of the protein have thus been examined. The solvent accessibility for hydrophobic residues present in the C-terminal domain are reported in Table I. The accessibility of at least four residues including L374, L424, F437 and F440 is significantly increased upon truncation, indicating that their side chains are exposed at the surface of the molecule. The three-dimensional model of the truncated dimer revealed that these hydrophobic residues are close to one another and form a hydrophobic cluster at each pole of the dimer (Figure 6). Whereas other interactions are not excluded, it is proposed that such an hydrophobic area might possibly support the subunit assembly in the L_8 mutant.

On this assumption, a hypothetical model of the L_8 mutant was elaborated by computer work (Figure 7). The model was built up by aligning the truncated dimers end-to-end so

that the hydrophobic patches exposed at each pole were close to one another and shielded from the solvent. In such a way, the proposed model showed a ring-like structure composed

of four dimers, in agreement with the micrograph analysis of the mutant protein. It can be noticed that this arrangement leads to a large central area as previously observed

Fig. 5. Negatively stained samples of Rubisco from mutant P441 (**top left**) and *Anacystis nidulans* (**top right**). Rotational analysis (Markham *et al.*, 1963) of a mutant particle is shown at the bottom.

(Figure 5). Furthermore, the size of the model is close to the dimensions deduced from the microscopic view of the L_8 mutant. This model gives an L_8 structure that differs from the arrangement of the dimers in the L_8S_8 Rubisco. In the L_8S_8 type, the dimers are arranged side by side whereas in the L_8 mutant the four dimers are arranged end to end.

Table I. Solvent accessibilities^a for hydrophobic residues in the C-terminal section of the Rubisco subunit from the native enzyme and mutant proteins truncated at positions P449 and P441, respectively

Residue	Accessibility ($\text{\AA}^2$)		
	Wild-type	Mutant	
		P449	P441
A373	0	0	0
L374	0	0	58
P377	17	17	26
G378	34	34	42
I400	98	98	98
A405	13	13	13
V421	53	53	53
P422	79	79	79
V423	10	10	27
L424	34	81	108
A434	18	35	35
F437	0	29	63
F440	27	27	89
P441	49	67	
A444	0	11	
Y448	29	89	

^aThe solvent accessibility was calculated using a program written by Kabsch and Sander (1983).

Finally, this working hypothesis was tested by the experimentation. The strategy was to engineer a polar residue into the hydrophobic cluster present in the truncated dimer. A L424 to N424 substitution was created from the plasmid coding for mutant P441. The choice of this change was driven by experimental convenience that allowed the use of the cassette method for this mutation. The recombinant protein was characterized by electrophoresis and immunoblot analysis as before (Figure 8). The double mutant P441/N424 had an electrophoretic mobility similar to the wild-type protein. This observation revealed that the L424 to N424 substitution restored the original L_2 type and demonstrated that the interactions within the L_8 structure of mutant P441 had thus been disrupted.

Catalytic and binding properties of the mutant protein

Significant levels of carboxylase activity were detected in one extract from the mutants. The truncated dimer E458 is as active as the wild-type enzyme, while the L_8 mutants P449 and P441 show a considerable loss of activity (Table II). Long reaction times were required to detect CO_2 fixation with the L_8 mutants, even when assayed at 6 mM RuBP. However, the carboxylase activity was inhibited by 2-carboxy arabinitol-1,5-bisphosphate (CABP), thus demonstrating the specificity of CO_2 fixation. CABP, an analog of the six-carbon reaction intermediate of the carboxylase reaction, is known to bind very strongly to the catalytic site of the activated enzyme (Pierce *et al.*, 1980). The inhibition of CO_2 fixation by CABP indicates that the active site of the mutant protein has retained at least some binding ability. The enzyme- CO_2 - Mg^{2+} -CABP complex has also been detected by use of radioactive markers. The quaternary complex was isolated by gel

Fig. 6. The $C\alpha$ backbone of the dimer of mutant P441 (left: side view; right: top view). Hydrophobic residues which are exposed upon truncation are indicated.

Fig. 7. Pictures of the L_8 mutant from *R. rubrum* (right) and the L_8 core from spinach Rubisco (left). The picture of the L_8 core of the spinach enzyme was generated from coordinates kindly provided by Drs Andersson and Knight. Side views of these models are shown at the bottom.

filtration of the L_8 mutant pre-incubated in the presence of [^{14}C] CO_2 , MgCl_2 and CABP. The level of carbamate bound to the mutant protein (0.83 mol/mol protomer) was close to the 1:1 stoichiometry found with the wild-type enzyme. However, the stability of the complex was greatly reduced relative to that of the wild-type enzyme. An excess of CABP in the elution buffer was necessary to prevent the dissociation of the quaternary complex during gel filtration. The additional mutation L424 to N424 did not improve the catalytic properties of mutant P441, despite the recovery of a dimeric structure. Loss of activity was also observed with the L_2 form of mutant P449 that was found in equilibrium with the L_8 in cell lysates (Figure 3).

Discussion

Site-directed mutagenesis was used here to examine the effects of truncation in the tail section of the enzyme subunit. Mutations in this area were selected on the basis of secondary structural motifs found in the subunit structure. Removal of the very last residues from *R. rubrum* Rubisco (Figure 2) allowed us to evaluate the significance in the homology between this C-terminal sequence (residues 456–466) and one of the conserved regions in the small subunit (residues 10–20). Neither the protein structure nor the enzymatic activity were found to be significantly altered in the resultant mutant E458. It is therefore unlikely that this C-terminal sequence functions as a small subunit substitute in those Rubiscos lacking small subunit. Additionally, this C-terminal

Fig. 8. Immunodetection of Rubisco from cell lysates of *E. coli* after electrophoresis as in Figure 3. *E. coli* cells contain wild-type Rubisco (lane 1) or mutant P441 before (lane 2) and after L424 to N424 substitution (lane 3).

peptide and the conserved region of the small subunits occupy quite different positions in the three-dimensional space, relative to their respective β/α barrels (Andersson *et al.*, 1989). Therefore, a statistical coincidence seems the most plausible explanation for this sequence homology.

Larger excisions in the tail domain had greater structural consequences. Removal of the last and penultimate α -helices

Table II. Specific activity of mutant Rubiscos

Mutant	Structure	Sp. act. (%)
E458	L ₂	88.0
P449	L ₈	0.1
P449	L ₂	0.2
P441	L ₈	0.1
P441/N424	L ₂	—

The wild-type enzyme has 100% carboxylase activity. The activity of the mutant P441/N424 is below the limit of detection.

significantly altered the quaternary structure of the protein. The truncated large subunits preferentially assemble into a polymer larger than the wild-type enzyme that was identified as an octamer by its molecular weight and 4-fold rotational symmetry. The apparent differences in the electron microscopic view of the L₈ mutant and *A. nidulans* enzyme, however, suggest some differences in the subunit packing. Our results suggest that the L₈ mutant protein has a different structure from that of the higher plant type L₈ cores. The removal of the terminal helices resulted in the exposure of shielded hydrophobic side chains (Figure 6). The solvent accessibility for some of the hydrophobic residues (L374, L424 and F440) was found to progressively increase upon truncation (Table I). The removal of helix J yielded an equilibrium of dimers and octamers (mutant P449), whereas removal of helix I and J yielded an octameric structure. Further deletions up to residues K431 or V423 increased the solvent accessibilities of hydrophobic residues and led to the complete aggregation of the subunits as insoluble mutant protein (data not shown).

Exposure of the hydrophobic surface to the solvent thus appears to be a plausible factor in the formation of an octamer from the truncated dimers. The disruption of the hydrophobic surface by a polar residue as seen with the L424 to N424 mutation regenerated the dimeric structure and confirms the above hypothesis. The exposed hydrophobic surfaces of the dimer interact with each other and result in an end-to-end packing of the molecules. The absence of intermediates such as L₄, L₆ and polymers larger than L₈ points to a closed structure rather than a linear polymerization. The model of the truncated L₈ mutant, as shown in Figure 7, satisfies these constraints. The L₈ configuration might also be a more stable form than larger polymers.

The 500- to 1000-fold decline in enzymatic activity of the L₈ mutant indicates some essentiality of the tail domain. Alteration of the active site by truncation of the large subunit was revealed by binding studies. In the case of the wild-type enzyme, addition of a slight excess of CABP is sufficient to form a stable quaternary complex which can be isolated. In the case of the L₈ mutant, the quaternary complex could only be demonstrated in the presence of a 100-fold molar excess of CABP. These results indicate that CABP is less tightly bound than in the case of the wild-type enzyme. The formation and the stoichiometry of the complex confirm, however, that the mutant performs the carbamylation required for the activation as the wild-type enzyme. The weak and reversible binding of the transition state analog indicates that the substrate binding site is altered.

The crystal structure of Rubisco from *R. rubrum* (Schneider *et al.*, 1986) shows that the tail section is distant from the active site. This tail section is in contact with the β/α barrel

Fig. 9. C α tracing of parts of the C-terminal domain of the Rubisco subunit from *R. rubrum*, starting with strand number 7 of the β/α barrel at the bottom. Salt bridges which are involved in the packing of the four C-terminal helices to each other, or helices and loops of the β/α barrel are included. The position of one of the phosphate binding sites at the active site is indicated by a ball.

and the question arises as to how mutations in this area are transmitted to the active site. Despite the fact that helix J is far from the active site, conformational changes can be mediated from this helix to the active site through a number of interactions (Figure 9). This helix is linked to the penultimate helix I through a salt bridge of R452 to D445. Helix I in turn packs against helix 7 of the β/α barrel through a salt bridge of D443 and R375. Additionally, there is a hydrogen bond from S439 of helix H to the main chain nitrogen of I400 as well as a salt bridge formed by R435 to D401. Both I400 and D401 are part of loop 8 in the β/α barrel, which forms one of the two phosphate binding sites. The removal of the terminal helix J destabilizes the packing of the helix I against helix 7. This destabilization can be mediated to the active site through helix 7 and the adjacent helix H (Figure 9). We conclude that the structural integrity of one of the phosphate binding sites relies on the structural support of the tail sections.

Conclusions

We have shown here that the removal of a few C-terminal residues from the *R. rubrum* Rubisco subunit completely changes the quaternary structure of the enzyme. The exposure of a hydrophobic patch results in the formation of an L₈ complex instead of the wild-type L₂ structure. This change in quaternary structure can be reversed by disrupting this hydrophobic patch through a single amino acid substitution in the truncated mutant.

Materials and methods

Chemicals

RuBP and CABP were synthesized as previously described (Weissbach *et al.*, 1956; Pierce *et al.*, 1980).

Plasmid and strain

A derivative of pRR 2119, the expression plasmid containing the *rbcl* gene coding for *R. rubrum* Rubisco (Somerville and Somerville, 1984), was used for mutagenetic experiments. A fragment bearing the *lacZ* and carboxylase sequences was introduced into a pUC118-type plasmid. As the recombinant Rubisco is fused to a 25 amino acid peptide derived from β -galactosidase at the N-terminus, the *lacZ* coding sequence upstream of the *rbcl* gene was removed and the resulting construct was designated as PL2. Restriction analysis performed on plasmid PL2 showed that the 5' end of the coding sequence was unaltered. Specifically the presence of *AvaII*–*MluI* and *AvaII*–*NcoI* restriction fragments was detected in the construct. The *AvaII* cleavage site is immediately after the start codon ATG. Moreover, the electrophoretic and kinetic properties of the recombinant Rubisco from PL2 plasmid expressed in *E. coli* were indistinguishable from the authentic *R. rubrum* Rubisco. *E. coli* strain JM83 was used as the host for the expression of *rbcl* gene.

Mutagenesis of the *rbcl* gene

Truncations from the 3' end of the *rbcl* gene were performed by site-directed mutagenesis. Synthetic oligonucleotides were designed to change codons for E458, P449 or P441 into stop codons. The mutagenic double-stranded oligonucleotides were introduced into the plasmid PL2 by replacement of a small fragment surrounding the site of mutation. A gap in the *rbcl* gene was created by digestion of the plasmid with restriction endonucleases (Figure 10). Unique restriction sites were exploited during the digestion of the plasmid. In addition to the restriction sites pre-existing in the wild-type gene, new sites were previously engineered by silent mutations to create short cassettes. The linear vector was isolated by agarose gel electrophoresis and subsequently ligated in the presence of the mutagenic insert. The re-circularized plasmid was directly used to transform competent cells by a CaCl_2 procedure (Maniatis *et al.*, 1982). Samples in which no insert was added in the ligation mixture were used as controls.

Plasmid DNA from ampicillin resistant colonies was isolated by the alkaline lysis procedure and screened by restriction site analysis. The mutagenic fragment that introduces a stop codon also destroys a restriction site within the cassette (Figure 10). Thus potential mutants were screened for the absence of one restriction site prior to DNA sequencing. The mutations were confirmed by sequence analysis of the *SphI*–*HindIII* region of plasmid DNA using a sequenase kit (US Biochemical Corp.). A 18mer oligonucleotide which primes 36 bases upstream from the *SphI* site was used during the analysis of the non-coding strand. Sequencing of the coding strand was performed from a M13 universal primer which primes 27 bases downstream from the *HindIII* site.

Expression and isolation of Rubisco

Inocula of *E. coli* harboring the wild-type or mutant constructs were grown in 2×YT medium containing 0.5% glycerol and 100 $\mu\text{g}/\text{ml}$ ampicillin, at 37°C under controlled agitation. After overnight growth, cells (5 g/l) were harvested by centrifugation (6000 g, 20 min) at 4°C, then frozen with liquid nitrogen and stored at –80°C.

The frozen cell paste was thawed in 50 mM Tris–HCl, pH 8.0 (5 ml/g cells) containing 1 mM DTT, 0.5 mM EDTA, 1 mg/ml lysozyme, 0.5 mg/ml DNase and 0.5 mg/ml PMSF. The cell suspension was kept at 30°C for 15 min and sonicated. More PMSF was added to the homogenate which was then heated at 50°C for 10 min. The extract was clarified by centrifugation (25 000 g, 10 min) at 4°C.

The supernatant was desalted by gel filtration on a Sephadex G-25, and the protein fraction was recovered in 50 mM Tris buffer, pH 8.0, 1 mM DTT and 0.1 mM EDTA (buffer A). The extract was applied to a Mono Q column (1.6 × 10 cm, Pharmacia), and eluted with a 400 ml linear gradient of NaCl (0–0.5 M) in buffer A. Fractions were analyzed by polyacrylamide gel electrophoresis (Phastsystem, Pharmacia) and Rubisco was identified by Western blot analysis using an immune serum raised against *R. rubrum* Rubisco.

Further purification of Rubisco was carried out by affinity chromatography. Carboxylase fractions were pooled and concentrated by ammonium sulfate precipitation. The protein pellet was dissolved in 50 mM Tris buffer, pH 8.0, desalted by filtration on a PD-10 column (Pharmacia) and applied to a column (1 × 10 cm) of green agarose (Amicon). The affinity column was run at room temperature as described by Soper *et al.* (1988). Alternatively, mutant Rubisco was purified by another additional step. After fractionation on Mono Q, the carboxylase extract was loaded to a Mono P column (0.5 × 20 cm, Pharmacia). The elution was developed with the same buffer system as before, using a 80 ml linear gradient of NaCl. Fractions containing the purified carboxylase were dialyzed against buffer A and concentrated on an ultrafiltration membrane (Amicon, PM10) before storage at –80°C in the presence of glycerol. Rubisco protein was determined at 280 nm (Schloss *et al.*, 1982).

Carboxylase and binding assays

Carboxylase activity was determined at 25°C by [^{14}C]CO $_2$ fixation assay (Lorimer *et al.*, 1977). The reaction mixture was composed of 25 mM [^{14}C]NaHCO $_3$ (0.5 $\mu\text{Ci}/\mu\text{mol}$), 10 mM MgCl $_2$, 0.75 mM RuBP in 100 mM Na-Bicine, pH 7.9. The reaction was started by the addition of enzyme pre-activated in 25 mM NaHCO $_3$ and 10 mM MgCl $_2$ for 30 min at 25°C. The assay was stopped by adding formic acid up to 10%. Aliquots of reaction mixture were removed and dried before liquid scintillation counting.

A solution of purified carboxylase was activated in 50 mM [^{14}C]NaHCO $_3$ and 20 mM MgCl $_2$ for 30 min at 25°C, before adding a 100-fold excess (over protomer) of CABP. After an additional 30 min, an aliquot was subjected to gel filtration on a column (1.6 × 15 cm) of Bio-gel P-10 (BioRad). Fractions were monitored for absorbance at 280 nm and radioactivity. The stoichiometry of CABP binding was calculated from the label of the protein fraction.

Electron microscopy

Carbon foil (4.8 nm thick, Arizona Carbon Foil Co., Tucson, AZ) cut into 3 mm squares with a scalpel, was floated off mica onto water. The squares were transferred via a bottom pickup onto 300 mesh copper grids that had been made sticky according to the method of Spiess *et al.* (1987). The carbon supports were glow discharged immediately prior to use. After adsorption onto the carbon for 25 s, the samples were washed with distilled deionized water and negatively stained with 0.5% uranyl acetate. Samples were micrographed using a Philips EM420 electron microscope operated at 120 kV.

Fig. 10. Encoded amino acid sequence and restriction map of the *rbcl* gene in the tail section of *R. rubrum* Rubisco. Boxes and underlined sequences have the same significance as in Figure 2. The restriction map shows the sites naturally occurring in the wild-type gene (above the line) and additional sites (below the line) introduced to create cassettes. Relevant features to the construction of the mutant genes are included.

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