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Comparison of the crystal structures of L_2 and L_8S_8 Rubisco suggests a functional role for the small subunit

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Comparison of the crystal structures of the L_2 and L_8S_8 forms of ribulose-1,5-bisphosphate carboxylase from *Rhodospirillum rubrum* and spinach respectively, reveals a remarkable similarity in the overall architecture of the L_2 building blocks in the two enzymes. Within the L subunits, no large conformational differences such as domain–domain rotations were found. In spite of a somewhat different packing of the L subunits in the L_2 dimer, the active sites of the two enzymes are highly conserved. Significant local conformational differences are, however, observed for the C-terminal part of the polypeptide chains as well as for loop 7, helix α7, loop 8 and helix α8 in the barrel domain. The small subunit forms extensive interactions with one of these α helices, α8, in the spinach L_8S_8 enzyme. The loops are at the active site and one of them forms a phosphate binding site for the substrate. We suggest that the small subunit modulates substrate binding and, possibly, the carboxylation/oxygenation ratio by inducing conformational changes in the active site through interactions distant from this site.

Key words: photosynthesis/protein crystallography/ribulose-1,5-bisphosphate carboxylase

Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase, Rubisco, catalyses the initial steps of two opposing metabolic pathways, carboxylation and oxygenation of ribulose-1,5-bisphosphate. The carboxylation reaction is the first step in the photosynthetic fixation of CO_2. The reaction yields two molecules of phosphoglycerate, which are partly recycled in the Calvin cycle to regenerate ribulose-1,5-bisphosphate, and partly converted to starch, the main storage form of photosynthetic chemical energy. The oxygenation reaction yields one molecule each of phosphoglycerate and phosphoglycolate. Phosphoglycolate is metabolized in the photorespiratory pathway where reduced carbon is oxidized to CO_2. The energy released in these reactions is dissipated as heat. Since the photorespiratory process causes considerable loss of photosynthetic energy in plants, Rubisco is an important target for protein engineering attempts to increase its carboxylation/oxygenation ratio.

Rubisco from most photosynthetic organisms, including plants and cyanobacteria, is a multisubunit complex consisting of eight large, L (55 kd) and eight small, S (14 kd) subunits. The enzyme from *Rhodospirillum rubrum*, in contrast, is a dimer of L-subunits. The amino acid sequence of the *Rh.rubrum* enzyme (Hartman *et al.*, 1984; Nargang *et al.*, 1984) shows 28% identity to that of the spinach L-subunits (Zurawski *et al.*, 1981) based on the structural alignment of the three-dimensional structures of the enzymes reported here.

Recent crystal structure determinations of L_2 Rubisco from *Rh.rubrum* (Schneider *et al.*, 1986a) and the L_8S_8 enzymes from spinach (Andersson *et al.*, 1989; Knight *et al.*, 1989) and tobacco (Chapman *et al.*, 1987, 1988) have shown that the basic functional unit for a Rubisco molecule is an L_2 dimer. Each of the two active sites in the dimer is built up from the C-terminal β/α barrel domain (residues 150–475) of one subunit and the N-terminal domain (residues 1-149) of the second subunit. Residue numbers refer to the spinach enzyme. This arrangement is reflected in regions of conserved active site residues which are present in both domains. These functionally equivalent residues have, however, different sequence numbers in bacterial and higher plant enzymes since insertions and deletions are present in several loop regions along the polypeptide chains. We therefore propose a unified numbering system for all Rubisco L-chains based on the alignment of the three dimensional structures of the enzymes from *Rh.rubrum* and spinach.

The core of the L_8S_8 enzyme consists of four L_2 dimers arranged around a molecular four-fold axis. The S-subunits are tightly packed between the tips of these elongated L_2 dimers, four at the top of the molecule and four at the bottom. Residues from the S-subunit do not interact directly with the active site in these L_8S_8 molecules. Nevertheless, the catalytic activity of the enzyme is modified by the S-subunits since removal of these subunits from the L_8 core decreases carboxylation activity by two orders of magnitude (Andrews, 1988). It is also possible that the S-subunits modulate the carboxylation/oxygenation ratio since all known L_8S_8 Rubisco molecules have a higher ratio than the L_2 enzyme from *Rh.rubrum* (Andrews and Lorimer, 1987). Clearly, elucidation of the functional role of the small subunits in the catalytic mechanism of L_8S_8 Rubisco is of importance for attempts to engineer Rubisco enzymes with different catalytic properties. We suggest here a mechanism by which the S-subunits influence the catalytic activity of the L-subunits in L_8S_8 enzymes.

Results and Discussion

The overall structures of Rubisco L subunits from Rh.rubrum and spinach are similar

In order to identify local structural differences between the *Rh.rubrum* and the spinach L subunits we independently aligned the two domains of the subunit. Superimposition of the N-terminal domains aligned 108 Cα atoms with a positional r.m.s. deviation of 1.1 Å. For the C-terminal domains the positional r.m.s. deviation was 1.6 Å for 236

Table I. Alignment of the amino acid sequences of Rubisco from spinach and *Rh.rubrum* based on the three-dimensional structure

```
1                10                  20                  30
M S P Q T E T K A S V G F K A G V K D Y K L T Y Y T P E Y E - - - - - T L D T D
                                     M D Q S S R Y V N L A L K E E D L I A G G E H
                                                    /βA/        &&αA&&&&&&&&     //

      40                  50                  60                  70
I L A A F R V S P Q P G V P P E E A G A A V A A E S S T G T W T T V W T D G L T
V L C A Y I M K P K A G Y G Y V A T A A H F A A E S S T G T N V E V C T - T D D
//////βB/////////          &&&&&&&&&&&&αB&&&&&&&&

      80                  90                  100
N L D R Y K G R C Y H I E P V A G E E N Q Y I C Y V A Y P L D L F E - - - - - -
F T R G V D A L V Y E V D E A - - - - - R E L T K I A Y P V A L F D R M I T D G
            ////βC/////                 /////βD//////

110               120                 130                 140
E G S V T N M F T S I V G N V F G F K A L R A L R L E D L R I P V A Y V K T F Q
K A M I A S F L T L T M G N N M G M G D V E Y A K M H D F Y V P E A Y R A L F D
     &&&&&&&&αC&&&&&&&&               ///////βE//////////     &&&&αD&&

150               160                 170                 180
G P P H G I Q V E R D K L N K Y - - - G R P L L G C T I K P K L G L S A K N Y G
G P S V M I S A L W K V L G R P E V D G G L V V G T I I K P K L G L R P K P F A
          &&&&αE&&&&&&&&               //β1/////                &&&&&&&&&

     190                 200                 210                 220
R A V Y E C L R G G L D F T K D D E N V N S Q P F M R W R D R F L F C A E A L Y
E A C H A F W L G - G D F I K N D E P Q G N Q P F A P L R D T I A L V A D A M R
&&&α1&&&&&&&&&          /β2//                  &&&&&&&&&&&&&&&&&&α2&&&&&&&

     230                 240                 250                 260
K A Q A E T G E I K G H Y L N A T A G T C E D M M K R A V F A R E L G - - - - -
R A Q D E T G E A K L F S A N I T A D D P F E I I A R G E Y V L E T F G E N A S
&&&&&&&&&&        //β3/             &&&&&&&&&&&&&&3&&&&&&&&&&&&&&&

               270                 280                 290                 300
V P I V M H D Y L T G G F T A N T T L S H Y C R D N G L L L H I H R A M H A V I
H V A L L V D G Y V A G A A A I T T A R R R F P - - D N F L H Y H R A G H G A V
  //β4///////       &&&&&&&&&&&α4&&&&&&&&&&&&&          /β5////        &&αF&

               310                 320                 330
D R - Q K N H G M H F R V L A K A L R L S G G D H I H S G T V V - G K L E G E R
T S P Q S K R G Y T A F V H C K M A R L Q G A S G I H T G T M G F G K M E G E S
&            βF/   &&&&&&&&&&&α5&&&&&&&&&&       /β6//

340               350                 360                 370
D I T L G F V D L L R D D Y T E K D R S R G I Y F T Q S W V S T P G V L P V A S
- S D R A I A Y M L T Q D E A Q - - - - - G P F Y R Q S W G G M K A C T P I I S
     &&&&&&a6&&&&&&&     //βG/                 //βH/              ////β7///

380               390                 400                 410
G G I H V W H M P A L T E I F G - D D S V L Q F G G G T L G H P W G N A P G A V
G G M N A L R M P G F F E N L G N A N V I L T A G G G A F G H I D G P V A G A R
/           &&&&&&&α7&&&&&&&          //β8///                  &&&&&&&&&&&

 420               430                 440                 450
A N R V A L E A C V Q A R N E G R D L A R E G N T I I R E A T K W S P E L A A A
S L R Q A W Q A W R D G V P - - - - - - - - V L D Y A R E H - - - - K E L A R A
&&&α8&&&&&&&&&&&&&&&&&&&&                  &&&&&&αG&&&&&&          &&&&&&&&&αH&

 460               470                 480
C E V W K E I K F E F P A M D T V
F E S F P G D A D Q I Y P G W R K A L G V E D T R S A L P A
&&&&&&&   &&&&&&αI&&&&&     &&&&αJ&&&&
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The secondary structural elements as found in the *Rh.rubrum* Rubisco are also included. First line: spinach sequence number, second line: spinach Rubisco sequence, third line: *Rh.rubrum* Rubisco sequence, fourth line: secondary structural elements, as found in *Rh.rubrum* Rubisco.

equivalenced Cα atoms. Based on these superimpositions we have aligned the amino acid sequences of Rubisco from spinach and *Rh.rubrum*. This alignment is given in Table I which we propose could form the basis for a unified numbering system of all Rubisco L chains using the spinach numbers as standard. Figure 1 shows the distance between equivalent Cα atoms as defined in Table I as a function of residue number. The Cα atoms that do not align are found in loop regions between secondary structure elements as well as in two regions of the structure, one in each domain (see discussion below).

Differences in packing of the domains within the subunit were analyzed by first superimposing the complete subunits using the transformation obtained from the alignment of the C-terminal domains alone. With this alignment, the r.m.s. deviation of the 108 Cα positions that were equivalenced in the experiment using only the N-terminal domains was 1.6 Å. To obtain the 'best' alignment an additional rotation of 2.5° was required. This difference in the packing of the domains is of the same order as that found for the two subunits of the *Rh.rubrum* enzyme. Thus, within the limits of our experiments, we conclude that there are no differences

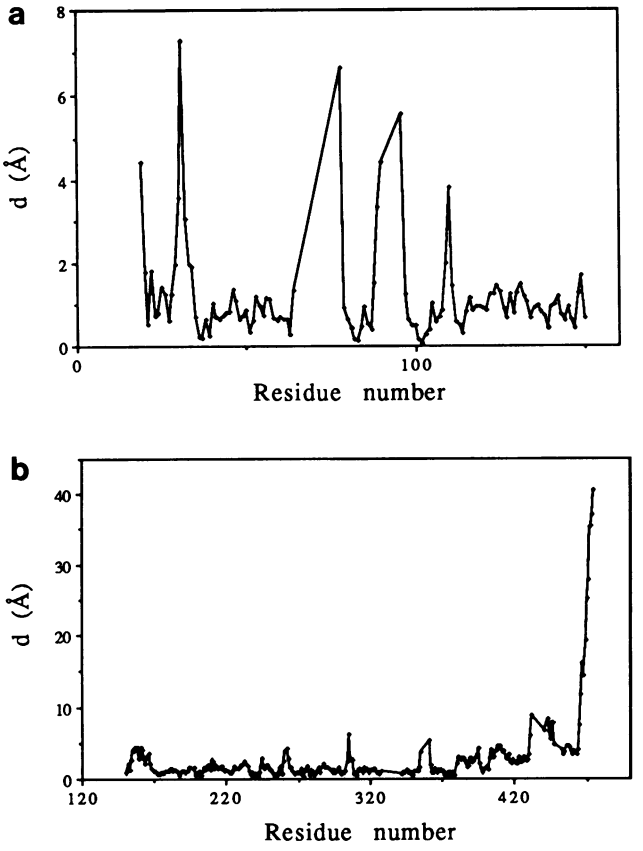

Fig. 1. Distance between Cα atoms from equivalent residues (Table I) in the L subunits of *Rh.rubrum* and spinach after alignment of (**a**) the N-terminal domain and (**b**) the C-terminal domain.

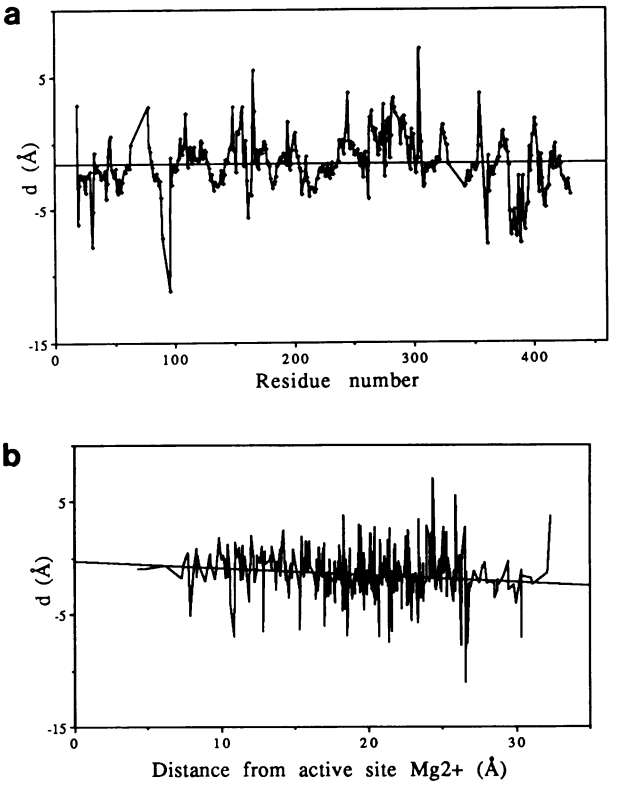

Fig. 2. Difference in the distance between symmetry related Cα atoms in the spinach and *Rh.rubrum* L_2 dimer $[(C\alpha'\text{-}C\alpha'')_{spinach} - (C\alpha'\text{-}C\alpha'')_{rubrum}]$as a function of (**a**) residue number and (**b**) distance to the closest active site magnesium ion. The first point with significant distance d to the active site metal ion is due to Gly 380 in loop 7 which has a different position in the two structures. The straight line in both figures represents a least-squares fit through the points.

in the domain−domain packing between the *Rh.rubrum* and the spinach L subunits.

The active sites are highly similar in the two forms despite differences in subunit−subunit packing in the L_2 dimers

If the two dimers are superimposed using the transformation obtained by alignment of one of the L subunits from each dimer, the other two L subunits do not superimpose. With this alignment the positional r.m.s. deviation for 345 Cα atoms in the second pair is 3.0 Å, in contrast to 1.6 Å for the same atoms after alignment of the subunits. An additional rotation of approximately 7° around an axis passing through the centre of gravity of the dimer and with direction cosines l = 0.0896, m = 0.1631, n = 0.9825 followed by a translation t = 0.4 Å along the rotation axis is required to align the second pair of L subunits. Similar results are obtained if only the C-terminal domains or the well defined β-strands in the β/α barrel are used in the initial alignment.

To examine the effect of this difference in the packing of subunits in the L_2 dimer we have plotted the difference in distance between symmetry related Cα atoms in each dimer as a function of residue number (Figure 2a) and as a function of the distance to the active site magnesium ion (Figure 2b). As can be seen from Figure 2a, the *Rh.rubrum* structure is more 'open' than the spinach structure. On average, symmetry related Cα atoms in the *Rh.rubrum* dimer are 1.5 Å further apart than the corresponding atoms in the spinach dimer. For some parts of the structure, notably the C-terminal extension, the difference is much larger. However, the active site is largely unaffected (Figure 2b) by the different subunit-packing in the *Rh.rubrum* and the spinach dimers. Thus, despite the differences in the packing of the two subunits, the active sites which are built up from residues from both subunits are highly similar in the two enzyme species.

Only a few conserved interactions are necessary to form the dimer

The interactions between the two subunits which form the L_2 dimers are of two types: interactions between the two C-terminal domains of the two subunits and interactions between the C-terminal domain from one subunit and the N-terminal domain from the second subunit. 40% of the amino acids that form the subunit−subunit interface are conserved. Besides the nonconserved residues in the hydrophobic core formed at the interface between the two C-terminal domains, there are eight hydrogen bonds in the spinach enzyme and four hydrogen bonds in the *Rh.rubrum* enzyme. None of these interactions are conserved.

Some of the interactions between the N- and C-terminal domains are of functional significance since they form part of the active site and involve residues which bind the active site metal atom or the substrate. However, since the spinach structure is of the activated enzyme with bound transition state analogue whereas the *Rh.rubrum* structure is of the non-activated enzyme, there are small but significant differences

Table II. Conserved interactions at the dimer interface*

Residues		Type of interaction
Thr 63A	Lys 177B	hydrophobic
	Leu 178B	hydrophobic
Leu 107A	Gln 209B	hydrogen bond
	Leu 178B	hydrophobic
Thr 118A	Glu 204B	hydrophobic
Asn 123A	Glu 204B	hydrogen bond

*: Only interactions between the C-terminal domain of one subunit and the N-terminal domain of the second subunit are listed.

in the conformation of some of the side chains involved in these interactions. Furthermore, many more residues are involved in this dimer interaction in the spinach enzyme due to amino acids found in conserved loop regions which are flexible in the *Rh. rubrum* enzyme but ordered in the spinach structure (see below). Obviously, these interactions are not important for the formation of a functional L_2 dimer. In total, we observe twelve interactions which are structurally conserved (Table II). These involve eight strictly conserved residues. In addition to these strictly conserved interactions, we find a conserved salt bridge between E 109 (D in *Rh. rubrum*) and R 253 as well as a main chain to main chain hydrogen bond between G 122 and M 297 (G in *Rh. rubrum*).

Structural differences between the enzymes

The Cα atoms that do not align between the two structures are mainly concentrated in two regions of the structure, one in each domain apart from a few amino acids in loop regions between secondary structure elements, where insertions and deletions are observed frequently. In the N-terminal domain helix A is absent in the spinach enzyme. In the bacterial enzyme this helix connects βA and βB of the five-stranded β sheet of the N-terminal domain and covers one side of the sheet. In the spinach enzyme the corresponding connection is instead an extended chain. As a consequence, this face of the β- sheet is more exposed to solvent.

The second non-aligned region comprises the last 40 residues of the C-terminal domain, which form a helical extension of the β/α barrel. The positions of these helices are different and two of them, αI and αJ, are not present in the spinach enzyme.

Two loop regions are of special interest. The loop that connects αB and βC in the N-terminal domain is flexible in the *Rh. rubrum* structure (Schneider *et al.*, 1986a), but is fixed in the spinach model (Andersson *et al.*, 1989). This loop forms a major interaction area with the small subunit and also participates in forming the active site. In the C-terminal domain there is also a flexible loop in the bacterial enzyme; the loop that connects β strand 6 with helix 6 in the β/α barrel. In the spinach enzyme this loop has a well defined conformation. The loop contains a conserved Lys residue, Lys 334, which reaches into the active site. The side-chain of this residue closes the entrance to the active site. Apparently these two loop regions are locked into one conformation in the activated spinach structure through interactions with the transition state analogue, CABP, which is not present in the non-activated enzyme.

A comparison of the β/α barrel domains shows some local conformational differences, which extend into the active site. The eight β strands, six of the helices and most of the connecting loops superimpose quite well (Figure 3). However, the remaining two helices α7 and α8 and the two loop regions which connect them with the β-strands in the active site region are significantly shifted in the spinach enzyme although the actual conformation of the loop regions appears to be quite similar. These shifts also propagate to the 40 residues of the C-terminal extension after the barrel (Figures 3 and 4).

The two loop regions connecting β strands 7 and 8 with

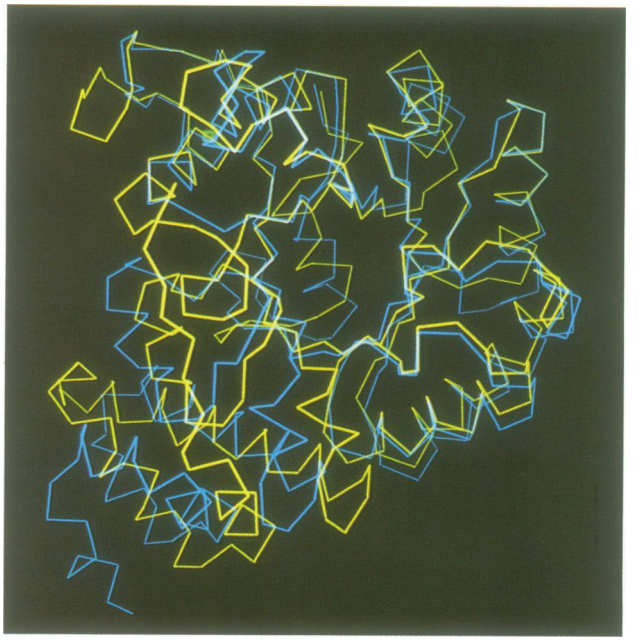

Fig. 3. Superimposition of the β/α barrel domains of ribulose-1,5-bisphosphate carboxylase from *Rh. rubrum* and spinach. The Cα tracing of the β/α barrel domain from the *Rh. rubrum* enzyme is shown in blue and for the spinach enzyme in yellow.

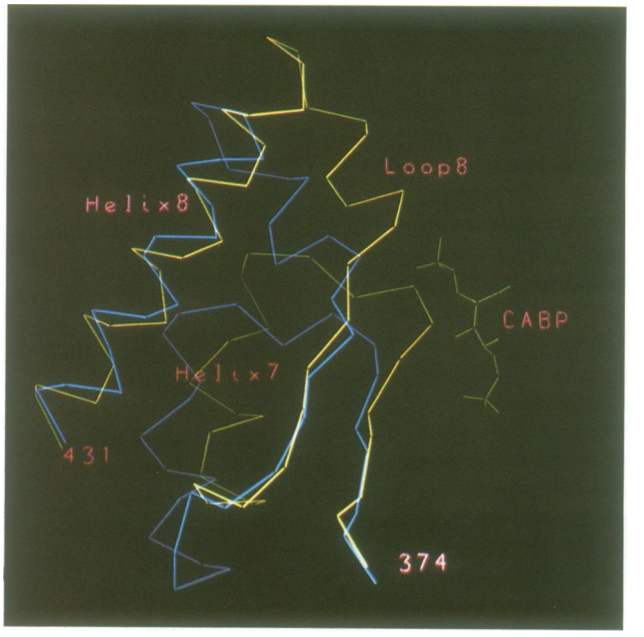

Fig. 4. Conformational differences at one of the phosphate binding sites in the active site of ribulose-1,5-bisphosphate carboxylase. The Cα atoms for the subunit of the *Rh. rubrum* enzyme are shown in blue, those from the spinach enzyme are shown in yellow. The bound inhibitor 2-carboxy-arabinitol-1,5-bisphosphate with one of its phosphate groups close to the helix in loop 8 is shown in yellow.

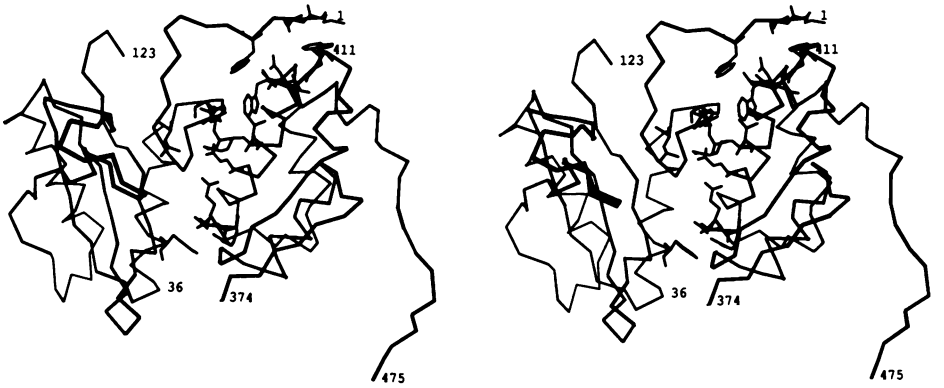

Fig. 5. Stereo picture of one of the interface regions between the large and small subunits of spinach ribulose-1,5-bisphosphate carboxylase. The side chains of conserved residues in higher plant carboxylases are included.

helices 7 and 8 are both part of the active site of the enzyme. Loop 7 contains a strictly conserved Ser residue, which is involved in inhibitor and product binding (Andersson *et al.*, 1989; Lundqvist and Schneider, 1989a,b). Loop 8 contains a short α-helix which is part of one of the phosphate binding sites at the active site (Figure 4). A shift of these parts of the active site will result in differences in substrate binding in the spinach versus *Rh.rubrum* enzyme.

Small subunits may modulate catalytic activity

In the spinach enzyme, parts of loop 8, helix α8 as well as the C-terminal extension are involved in the interactions of the large subunit with the small subunit (Figure 5). This extensive interaction area comprises 14 side-chains from the L and 13 side chains from the S subunits. These residues are listed in Table III. Most of them are strictly conserved in all higher plant carboxylases, but are quite different in the *Rh.rubrum* enzyme. In particular, helix 8 interacts extensively with the N-terminal arm of the small subunit. The C-terminus of this helix also interacts with a second small subunit. The conformation of the C-terminal tail section as observed in the L_2 *Rh.rubrum* enzyme is not possible in the L_8S_8 complex, since it would lead to close contacts of residues 429, 433, 448 and 449 from the L chain with residues 15, 18, 19 and 29 from the small subunit (Figure 6). We suggest that the small subunits induce the observed shift of helices α7, α8, their preceding loops and the C-terminal section of the L chain in relation to the β/α barrel. It is conceivable that these conformational changes could influence catalytic activity and, possibly, the partitioning between carboxylation and oxygenation. Indeed, a weaker binding of CABP to the L_8 core compared to the L_8S_8 enzyme of Rubisco from *Synechococcus* has been observed (Andrews, 1988). As a consequence, the design of mutagenesis experiments in order to improve the carboxylation/oxygenation ratio of the plant enzyme may have to consider mutations in both the large and the small subunits.

From these studies, we cannot exclude the possibility that some of the observed conformational differences are due to ligand binding in the quaternary complex of the spinach enzymes. However, binding of CABP to non-activated *Rh.rubrum* enzyme does not change the conformation of these parts of the β/α barrel in the crystal (Lundqvist and Schneider, 1989b). Since the conformation observed in the *Rh.rubrum* enzyme is prevented by steric hindrance from the small subunits in the spinach enzyme, we find it reasonable to conclude that these conformational differences are induced by the small subunits which thereby modulate the activity of the large subunit.

Table III. Interactions between the small & large subunits of spinach Rubisco

Spinach large subunit	Spinach small subunit
*D397	R108
*W411	*M1, *Q2
*G412	*L72
*A414	*W4
V418	*W4
*R421	*Y17
*V422	*Y17
*E425	*Y17, *L18
Q429	*L18, *L21
*R431	*Y32
*N432	*Y32, E29
*E433	Q25, A28
*W451	*L18, *P19
*E454	*W4

Interactions between the small and large subunits of spinach Rubisco that involve residues from β-strand 7 in the barrel domain to the C-terminus of the polypeptide chain. Only side-chain interactions within 4.0 Å are given. Conserved residues in the higher plant carboxylases are marked by *. For structurally corresponding residues in the *Rh.rubrum* enzyme see Table I.

Conclusions

We have compared the three-dimensional structures of two very different Rubisco molecules, one which is from a photosynthetic bacterium and which is only an L_2 dimer and one from a higher plant where four L_2 dimers are glued together by eight small subunits into an L_8S_8 molecule. The large subunits of these molecules show only 28% amino acid sequence identity. We find a surprising similarity in the overall structure of L_2 dimers in these two molecules. We also find small local conformational differences which seem to be due to the presence of small subunits in the L_8S_8 enzyme. These differences extend into the active site and modulate details of the substrate binding site with possible functional consequences.

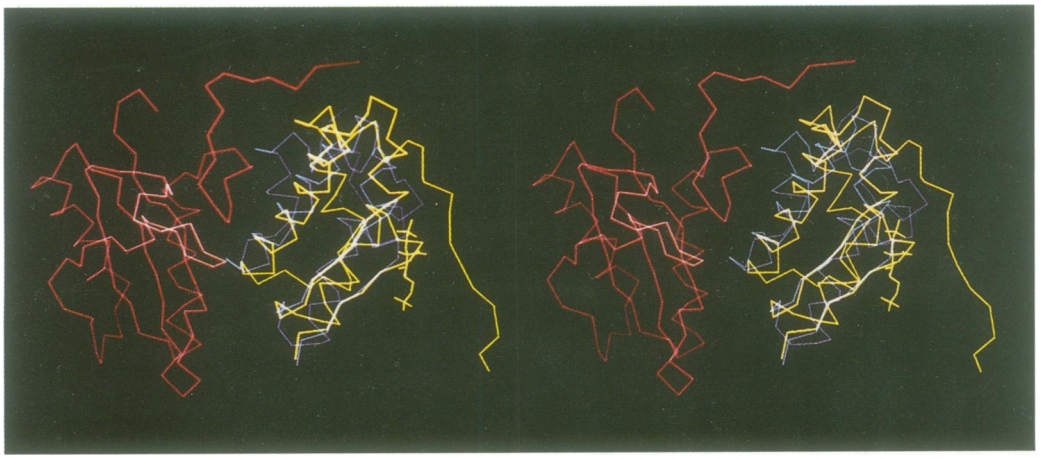

Fig. 6. Stereo picture of the interface between the small and large subunits of the spinach ribulose-1,5-bisphosphate carboxylase. The corresponding parts of the *Rh.rubrum* Rubisco are superimposed. Colour coding: blue: *Rh.rubrum* subunit, yellow: spinach L subunit, red: spinach S subunit. The bound inhibitor 2-carboxy-arabinitol-1,5-bisphosphate is also shown in yellow.

Materials and methods

Structural comparison of the L_2 units in Rubisco from *Rh.rubrum* and spinach was made using Cα atoms from refined models of the two structures (Schneider *et al.*, 1986a; Andersson *et al.*, 1989; Knight *et al.*, 1989). The molecular structures are presently refined at a resolution of 1.7 Å for the *Rh.rubrum* enzyme and 2.4 Å for the spinach enzyme with crystallographic R-factors of 18.0% and 19.0%, respectively. The structure of the *Rh.rubrum* enzyme represents the non-activated form of the enzyme (Schneider *et al.*, 1986b), whereas the spinach Rubisco model is of the activated quaternary complex with a bound transition state analogue, 2'- carboxy-D-arabinitol-1,5-bisphosphate, CABP (Andersson and Brändén, 1984).

The two subunits of the *Rh.rubrum* enzyme are related by an approximate two-fold axis. Due to different environments in the crystal, the two L subunits differ in the relation between the two domains. If the two C-terminal domains are superimposed, an additional rotation of 3° is required to obtain the best least-squares fit between the two N-terminal domains. Furthermore, the subunits in the *Rh.rubrum* L_2 dimer show a 4° deviation from two-fold symmetry for the last 40 amino acid residues in the C-terminal extension. The tilt of the C-terminal region causes a deviation from two-fold symmetry also for the adjacent parts of the β/α barrel i.e. helices α7, α8 and loop 8. The conformational differences in loop 8 between the two subunits is even larger than explained by the tilt of the C-terminal tail region and probably reflects conformational flexibility in the unliganded enzyme. One of the subunits shows more rigidity in this area as judged from the B-factors, probably due to tighter packing in the crystal. This subunit also gives the best alignment with the spinach L subunit. For subunit−subunit alignments of the two different enzymes, results based on this *Rh.rubrum* subunit are reported. Alignment based on the second subunit leads to the same conclusions.

Superimposition of the *Rh.rubrum* model and the L-subunits of the spinach enzyme was made by least-squares methods using the program O (Jones *et al.*, 1990). A small set of core atoms in each domain was used for an initial alignment which was subsequently used to maximize the number of structurally homologous Cα atoms in each domain. Atoms were considered to be equivalent if they were within 3.8 Å distance from each other within a consecutive region, consisting of at least four residues.

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