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REVIEW

INVESTIGATIONS ON THE STRUCTURE AND CONFORMATIONAL DYNAMICS IN RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE (RUBISCO) MOLECULE

Klimentina Demirevska-Kepova*, Ljudmila Simova-Stoilova

Acad. M. Popov Institute of Plant Physiology, Acad. G. Bonchev Str., Bl. 21, Sofia 1113, Bulgaria

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Summary. A review is made of the published investigations concerning the structure and conformational dynamic changes in the key photosynthetic and photorespiratory enzyme – Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) of higher plants. Recent data on the basis of X-ray crystalography, NMR-spectroscopy, site-directed mutagenesis and other techniques are analysed. Despite some differences in the aminoacid sequence of Rubisco from various species, significant conformational changes are not established. The tertiary and quaternary structure of Rubisco is described in detail emphasizing the active and regulatory sites of the enzyme. Possibilities and limitations of the different methods of studying protein structure and conformation are discussed. Some advantages of the immunochemical methods especially the use of monoclonal antibodies for investigations of dynamic changes of protein structures in solution are pointed out.

Key words: Rubisco, structure, conformational dynamics, immunochemical approaches

Abbreviations: ATP-ase – adenosine-5'-triphosphatase; CABP – carboxyarabinitol bisphosphate; LS – large subunit; RuBP – ribulose-1,5-bisphosphate; SS – small subunit

Rubisco is the initial enzyme of both photosynthetic and photorespiratory metabolism in autotrophic plant leaves, which catalyses the carboxylation of Ribulose bisphosphate

*Corresponding author

(RuBP) in Calvin's photosynthetic pentosephosphate pathway and the oxygenation of the same substrate in the photorespiratory pathway (Lorimer, 1981; Keys, 1986; Walker et al., 1986). The ratio between the two metabolisms as well as the amount and activity of the enzyme largely determine the photosynthetic efficiency and productivity of plants. That is why Rubisco is an attractive target for future protein engineering in attempts to create plant species with improved efficiency of carboxylation and decreased photorespiration (Ellis and Gatenby, 1984; Somerville, 1986). More detailed analyses of the structure and the conformational dynamics of the enzyme's molecule are required for further investigations in this field.

Oligomer structure

Rubisco of higher plants is a large protein (hexadecamer) with a molecular weight of 560 kDa. Rubisco's molecule is a 16 subunit oligomer built from eight large (51–58 kDa) subunits (LS) and eight small (12–15 kDa) subunits (SS). Large subunits are synthesized in the chloroplasts and small subunits are synthesized in the cytoplasm first as precursors, which are then transported through the chloroplast membrane (Chua and Schmidt, 1978; Ellis, 1981; Ellis, 1987; Luben et al., 1988). Rubisco is a specific chloroplast enzyme, which is assembled in the chloroplast stroma, and is under dual chloroplast-nuclear genetic control.

In 1980 another nuclear-encoded high molecular chloroplast protein (Rubisco binding protein) with molecular mass 720 kDa was identified. This protein was found to have an auxiliary function in the correct assembly of the holoenzyme, as a chaperone, forming an intermediate complex with the Rubisco large subunits on the basis of complementary interactions (Gatenby, 1988; Hemmingsen et al., 1988; Roy and Cannon, 1988; Roy et al., 1988; Ellis et al., 1989; Roy, 1989, 1993). Crafts-Brandner and Salvucci (1994) reported the existence of Rubisco complex protein (120 kDa) which binds to the small subunit precursors in the cytosol and reduces their transport into the chloroplasts thus regulating the Rubisco holoenzyme assembly. There is also evidence that the light activation of Rubisco *in vivo* is performed by another specific chloroplast enzyme, i.e. Rubisco-activase (200 kDa), possessing ATP-ase activity. Rubisco-activase increases the proportion of the activated Rubisco, promoting the dissociation of tightly bound sugar-phosphates from the active site of the enzyme and probably induces conformational changes (Salvucci et al., 1985; Portis et al., 1986; Liley and Portis, 1990; Portis, 1990; Wang and Portis, 1992). There exist data about the participation of Rubisco in stable CO_2 -fixing multienzyme complexes with other enzymes from Calvin's cycle (Suess et al., 1993), suggesting conformational possibilities of association with other proteins. The exact mechanism of the Rubisco assembly and its regulation are still unclear.

Crystallographic data and molecular models

Most studies on the structural organisation of Rubisco from various plant species (mainly from tobacco and spinach) have been conducted using X-ray crystallographic analyses (Baker et al., 1975, 1977; Anderson and Branden, 1984; Nakagawa et al., 1986; Chapman et al., 1987, 1988; Lundqvist and Schneider, 1988; Knight et al., 1989). It has been established that Rubisco subunits are organised simetrically in the L_8S_8 octameric structure.

The complete sequence of the small subunits from *Spinacia oleracea* has been determined and shown to consist of a single chain of 123 residues (Martin, 1979). Small subunit sequences from other species have been obtained and close structural homology have been found (Mazur and Chui, 1985; Fluhr et al., 1986; Matsuoka et al., 1987). The large subunit amino acid sequence from *Zea mays* comprising 475 amino acid residues has been described by McIntosh et al. (1980). Later, data about the sequence of large subunits from other species have been reported (Shinozaki and Sugiura, 1982; Zurawski et al., 1981, 1986). Homology of 80% of the large subunits from higher plants was observed. Regardless of some differences in the aminoacid sequences, large conformational changes have not been established even for such evolutionary distant species as spinach and the photosynthetic bacterium *Rhodospirillum rubrum* (Schneider et al., 1990). The quaternary structure of spinach Rubisco typical for all higher plants and most bacterial carboxylases, has been analysed with highest resolution – 2.4 Å (Knight et al., 1990).

Branden et al. (1986) based on X-ray structural studies suggested an arrangement of LS in L_2 dimers of the L_8 molecule in a cloverleaf- or blockhouse fashion. The L_8 molecule thus obtained is ellipsoidal with a large hole in the middle arround the four-fold axis. The small subunits are placed in S_4 clusters on the top and on the bottom of the L_8 structure. The model of the L_8S_8 molecule is similar to that proposed by Bowien et al. (1980) who have used electron microscopy.

The tertiary structure of Rubisco from *Nicotiana tabacum* including domains and their contacts was determined by Chapman et al. (1987, 1988). The 474 residues of the L chain form two domains, each having long COOH-terminal extensions. The first 168 residues are the N domain. The residues from 169 to 474 are the B domain. The main domain (B) of L is an α/β barrel containing most of the catalytic residues. The active site is in a pocket at the opening of the barrel and is partially covered by the N domain of a neighbouring L chain. The 123 residues of the S chain fold into a brain-shaped domain with a COOH-terminal tail. According to the authors the pattern of intersubunit contacts is rather complex. Each large subunit contacts with four LS and three SS and each small subunit contacts with two SS and three LS. The interface areas between subunits bury almost half of the surface areas of both the large and the small subunits (Knight et al., 1990).

Knight et al. (1990) give full description of spinach Rubisco structure. The L_8S_8 molecule is cube-shaped with round edges and side of approximately 105 Å (10.5 nm). The molecule has 4-fold axis which relates four L_2 dimers into a core of eight large subunits $(L_2)_4$. The small subunits are arranged in two separate clusters of four subunits each $(S_4)_2$ which interact with the large subunits. Each small subunit binds in a deep crevice formed between the tips of two adjacent elongated L_2 dimers at each end of the L_8S_8 molecule. In the centre of the molecule there is a solvent channel, 1.5–3 nm in diameter.

According to Knight et al. (1990), LS of spinach Rubisco has two clearly separated domains (N and C). The N domain on LS (1–150 residues) is folded into a central mixed five-stranded β-sheet with two α-helices on one site of the sheet. C domain is α/β barrel (157–475 residues) and was found as a structure of many enzymes. The barrel represents 8 β-strands which form the core of a barrel surrounded by the 8 α-helices. Loops of aminoacid sequences connect the β-strands to the α-helices. The majority of aminoacid residues involved in catalysis and substrate binding are situated in the C-terminal loops (connecting the C-termini of the β-strands to the N-termini of the α-helices). The N-terminal loops are involved in subunit interactions in the L_8S_8 molecule.

The 123 amino acid residues of the SS are arranged in a four-strand anti-parallel β-sheet, covered on one side by two α-helices. The first 20 residues at the N-terminal form an irregular arm, which extends to a neighbouring SS, forming an interacting area between SS. Thus the S_4 clusters are organised.

The active site is located at the intra-dimer interface between the C-terminal domain of one LS and the N-terminal domain of the second LS and it is formed by two different LS (L_2 dimer). In fact, there are eight active sites in one L_8S_8 molecule on the external part of the cube-like structure. The sites of activation, of substrate binding and of some amino acids participating directly in catalytic reaction have been determined. In the C-terminal of the α/β barrel of the C domain of LS in loop 2, the activating site of the enzyme containing Lys, Asp, Glu has been established. Arg which binds one of the phosphate groups of the substrate, and His, which is involved in catalysis have been found in loop 5. Loop 6 is flexible, has an open and closed conformation and is very important for enzyme activation. Ser binding RuBP and the product or the reaction as well as carboxyarabinitol bisphosphate (CABP) is located in loop 7. Loop 8 (Gly-riched helix) is the second phosphate binding site. In the N domain there are sites which participate also in substrate to enzyme binding.

Conformational dynamics

As known both carboxylase and oxygenase functions of Rubisco are effected in the same catalytic site (Andersson et al., 1989). LS is responsible for the catalytic activity and some of its steps could be modulated by SS. According to Andrews (1988) SS

influences the catalytic activity of the enzyme increasing k_{cat} of the carboxylase reaction more than 100-fold. Schneider et al. (1990) suppose that SS modulates the active site of the enzyme and probably influences carboxylation/oxygenation ratio inducing conformational changes in the active site through interactions between sites far from it.

Activation or inhibition of the enzyme by different compounds before RuBP carboxylation or oxygenation is common for all Rubisco molecules. Rubisco is activated *in vitro* by CO_2, Mg^{2+} and by some metabolites. In the activation process one of the Lys residues is carbamilated by the CO_2 molecule, which is both the effector and one of the substrata of carboxylase reaction (Lorimer et al., 1980). Hence CO_2 molecule binds at two different sites on the Rubisco molecule. The mechanism of *in vitro* Rubisco activation is not fully understood (Ogren et al., 1986), but all the effectors influence directly or indirectly the active site of the enzyme.

Both carboxylase and oxygenase activities of Rubisco are influenced by structural changes in the catalytic and activation sites of the molecule (Pierce and Reddy, 1986). The quaternary complexes of the enzyme with CO_2, Mg^{2+} and CABP, have been studied spectroscopically using a variety of physico-chemical techniques. The crystal structure of the inactivated Rubisco from *Nicotiana tabacum* complexed with CABP (an analog of the transition state of the enzyme-substrate complex) has been determined and it has been found that CABP binds at the active site of the enzyme in extended conformation (Znang et al., 1994). Loop 6 is open and flexible until the analog binds and is closed in the activated enzyme complex. There are data suggesting the possibility of RuBP binding not only on the catalytic site of the enzyme but also at another non-catalytic site, which is probably related with the metabolic regulation of the enzyme (Yokota et al., 1994). Conformational changes in activated Rubisco are induced by Yokota et al. (1993) utilising the effect of sugar phosphates binding at the active site of the enzyme. Beuttemmulller et al. (1990) using polyclonal antibodies against Rubisco from *Nicotiana tabacum* have proven that the structure or the conformation of the enzyme of the mutant type with higher oxygenase activity is changed as compared to that of the enzyme of the wild type. There are scarce data about Rubisco oxygenase reaction and the mechanisms of its regulation (Schneider et al., 1992).

Immunochemical approaches

The precise structural models of proteins and their three-dimentional configuration obtained by X-ray crystallography describe the static state of the proteins without however considering the protein molecule dynamics, which is essential for understanding the functions of proteins in solution. It is not always possible to obtain crystal complexes of the enzyme with substrates or effectors. The function of some aminoacid residues of the Rubisco molecule have been investigated by site-directed mutagenesis (Haining and McFadden, 1990; Mural et al., 1990; Gutteridge et al., 1993; Zhu and

Spreitzer, 1994), which could not be used for studing Rubisco from higher plants because of specificities of the Rubisco assembly. The conformational flexibility of some regions of the protein molecule may have a substantial role in its biological function (catalytic or noncatalytic). For example, substrate binding to the active site of the enzyme or activation in other sites of the molecule may lead to conformational reorganizations during catalysis, which are impossible to study by such methods. Immunochemical approaches may be used to overcome this difficulty . Especially monoclonal antibodies with their high specificity and sensitivity to one determined site (one epitope) of the protein molecule (Vora, 1985) are suitable for evaluating the interactions between proteins and for investigating small but essential conformational changes in proteins at different steps of the activation and catalytic processes. Generally, the more active and flexible regions on the protein molecule are highly immunogenic and the size of the antigenic determinant is in order of a few aminoacid residues. It has been estimated, on the basis of the size of the antibody arm that epitops belonging to two monoclonal antibodies able to bind simultaneously to an antigen should be at least 35 Å apart (Wilson et al., 1984). All this makes monoclonal antibodies suitable reagents for study of fine structural differences and conformational changes.

Three monoclonal antibodies against Rubisco from the marine alga *Bryopsis maxima* (Kajikawa et al., 1988), seven monoclonal antibodies against Rubisco from *Nicotiana plumbaginifolia* (Meyer et al., 1991) and six monoclonal antibodies against Rubisco from *Hordeum vulgare* (Mladjova et al., 1992) have been obtained. Meyer et al. (1991), using competitive ELISA arrange the monoclonal antibodies against *Nicotiana plumbaginifolia* in three groups depending on the sites of recognition (epitope mapping). One of the groups reacts with Rubisco from dicotyledonous plants only. Comparison of the amino-acid sequences of Rubisco LS from monocotyledonous and dicotyledonous species did not reveal differences specific for the dicotyledonous only which was difficult to explain and the authors suppose that this group of monoclonal antibodies recognize the more flexible, more immunogenic but also less conserved region in Rubisco molecule.

In our investigations, the monoclonal antibodies against Rubisco from barley (Mladjova et al., 1992) separated in two groups: one recognising Rubisco from monocotyledonous and dicotyledonous and the other recognising Rubisco from monocotyledonous plants only. A cross-reaction was established between Rubisco and Rubisco binding protein in the group of monoclonal antibodies specific for Rubisco from monocotyledonous and dicotyledonous plants. Such cross-reaction was not found in the other group. We suggest that common determinants in the molecules of Rubisco and Rubisco binding protein exist, probably related to the function of Rubisco binding protein in the Rubisco holoenzyme assembly. Such cross-reaction was observed by Musgrove and Ellis (1986), Hemmingsen and Ellis (1986), Demirevska-Kepova et al. (1993) with polyclonal antibodies against Rubisco and Rubisco binding protein. Further investigations could explain the different results.

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