

Structural mechanism of RuBisCO activation by carbamylation of the active site lysine

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Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is a crucial enzyme in carbon fixation and the most abundant protein on earth. It has been studied extensively by biochemical and structural methods; however, the most essential activation step has not yet been described. Here, we describe the mechanistic details of Lys carbamylation that leads to RuBisCO activation by atmospheric CO₂. We report two crystal structures of nitrosylated RuBisCO from the red algae *Galdieria sulphuraria* with O₂ and CO₂ bound at the active site. *G. sulphuraria* RuBisCO is inhibited by cysteine nitrosylation that results in trapping of these gaseous ligands. The structure with CO₂ defines an elusive, preactivation complex that contains a metal cation Mg²⁺ surrounded by three H₂O/OH molecules. Both structures suggest the mechanism for discriminating gaseous ligands by their quadrupole electric moments. We describe conformational changes that allow for intermittent binding of the metal ion required for activation. On the basis of these structures we propose the individual steps of the activation mechanism. Knowledge of all these elements is indispensable for engineering RuBisCO into a more efficient enzyme for crop enhancement or as a remedy to global warming.

nitrosylation in algae | activity control | intermediate trapping

Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (EC 4.1.1.39) is a dominant contributor to conversion of gaseous CO₂ into biomass (1). Aerobic life forms, including heterotrophs, primarily derive their carbon through an autotrophic/ photosynthetic route using CO₂ as a carbon source. RuBisCO accomplishes this task by incorporating CO₂ into a phospho-sugar, ribulose 1,5-bisphosphate (RuBP). Incorporation of CO₂ into RuBP generates two molecules of 3-phosphoglycerate. This simple compound is subsequently used to build other organic molecules of life. Synthesized RuBisCO does not have a fully functional active site (2–4). It needs to be activated by a CO₂ molecule that carbamylates its catalytic Lys to bind Mg²⁺ that completes the activation process (5, 6). The mechanism of RuBisCO activation by the CO₂ molecule is presented in this work. This knowledge will contribute to acceleration of the progress in remodeling the RuBisCO for practical uses (7–10).

RuBisCO is present in many photosynthetic organisms including bacteria, algae, plants (2–4), and archaea (11). It accounts for roughly a half of the soluble protein mass in C3 plant leaves (1). However, RuBisCO is an inefficient enzyme with a low, 1–10/s, turnover rate (12). A side reaction with O₂ reduces its functional activity even further. The side reaction happens when a promiscuous enediolate intermediate of RuBP is attacked by O₂ (versus CO₂) and produces 2-phosphoglycolate (13). This reaction drains a pool of RuBP and decreases the efficiency of carbon fixation by up to 50%. The efficiency of RuBisCO is thus dependent on the ability to discriminate between CO₂ and O₂ or on the specificity ratio. The specificity ratio *S* is defined as: *S* = *V*_c*K*_o/*V*_o*K*_c, where *V*_c and *V*_o are maximal velocity for carboxylation and oxygenation, and *K*_c and *K*_o are the relative Michaelis constants for CO₂ and O₂, respectively. The specificity ratio has implications for crop yield, nitrogen and water utilization in plants, and the global carbon cycle. We propose the principles of substrate selectivity and suggest how to improve it.

Mutational studies produced a limited success in improving the catalytic efficiency of RuBisCO (9, 14, 15). This limited success stems from an incomplete understanding of the complex mechanistic principles of RuBisCO activation and catalysis (16). The inefficiency of the enzyme, however, does not affect its cellular function; therefore, it has been recently postulated that RuBisCO has evolved as an inefficient enzyme (17, 18), which is compensated by its abundance. Our work delineates the fundamental mechanism of RuBisCO activation by carbamylation and suggests the principles of gaseous ligand discrimination (O₂ versus CO₂) by the enzyme. As a result, we provide a road map for the potential improvement of the enzyme's selectivity and catalytic efficiency.

Forms of RuBisCO. The RuBisCO family represents a diverse group of enzymes. There are four organizational forms of RuBisCO: *I*, *II*, *III*, and *IV* (13, 19). Representative X-ray structures of these forms (19) are now available. The minimal functional enzyme is a homodimer, composed of two large subunits (L2). An individual subunit contains a catalytic, TIM-like barrel and a small C-terminal α+β domain. Frequently, RuBisCO contains two large subunits (L, 50–55 kDa) that are associated with two small subunits (S, 12–18 kDa). The most common form *I* is a L8S8 hexadecamer (Fig. 1). This form is present mostly in chemoautotrophic bacteria, cyanobacteria, red and brown algae, and all higher plants. Form *I* RuBisCO has four subtypes: *A* and *B* from cyanobacteria, eukaryotic algae, and higher plants and *C* and *D* from nongreen algae and phototropic bacteria (red-type enzymes). The form *II* enzyme is an L2 dimer of large subunits only. Form *III* is diverse in composition (L2, L8, and L10) but does not contain small subunits. Form *IV* encompasses RuBisCO-like proteins from organisms that do not use CO₂ as a major carbon source (20). The focus of this study is the L8S8 hexadecameric form *ID* of RuBisCO with a molecular weight of ~0.6 MDa from red algae *Galdieria sulphuraria* (21).

Results and Discussion

Structure. The enzyme was crystallized in the presence of ammonium sulfate (~2 M) and 2 mM Mg²⁺. The data were collected on lens-shaped crystals (21), characterized by the I422 space group, to ~1.9 Å resolution. The structure was solved by molecular replacement using the tobacco structure (3RUB) (22) as a template. The asymmetric unit contained a dimer of the small subunit (138 aa) and the 21–475 residues of a large subunit (493 aa) with the *N* and *C* termini disordered (L1S1). This constituted 1/8 of the

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 4F0H, 4F0K, and 4F0M).

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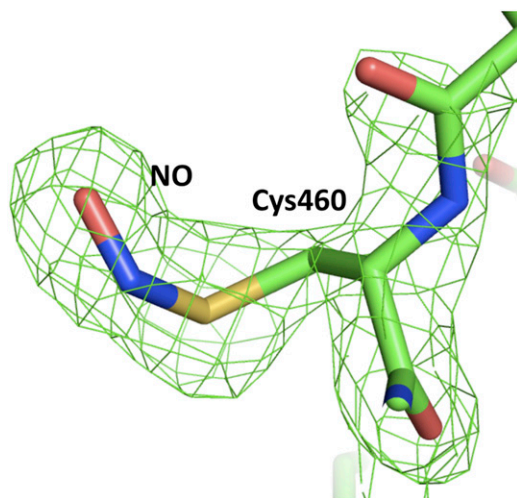


Fig. 2. The bond-stick model of the nitrosylated Cys460 covered with the omit map electron density (3σ).

were refined at Cys460 and Cys181, albeit, with higher temperature factors compared with Cys460, thus suggesting partial disorder. Both Cys residues have been implicated in the inactivation of RuBisCO by oxidation (26). The sequence 175-GRPLLGCTIKPKLGLS-190 is highly homologous in *G. sulphuraria* and green plants RuBisCOs.

This sequence includes the active site Thr182 and the highly conserved *cis*-Pro185 (in KPK motif, in boldface type above). This sequence also contains highly conserved Cys181, the presence of which correlates with inhibition by nitrosylation in several RuBisCOs investigated so far. The nitrosylation of Cys181 that is adjacent to Thr182 must cause a steric disturbance and marked change in the electrostatic signature of the active site. These changes must influence in a detrimental manner the unstable character of the activation complex making the carbamylation inoperable. Nitrosylation of Cys-460 was also implicated in this inhibition (26). Nitrosylation of Cys residues is a frequent posttranslational modification in higher eukaryotes; however, this modification has not been reported in algae (27), and thus the presence of nitrosylated Cys in *G. sulphuraria* RuBisCO is a unique finding.

The active site showed an increased disorder (mobility) that included the solvent as well as the protein (3) (Fig. 1 and Figs. S2–S4). Despite the increased mobility (higher Bs) in the active site region, we detected two sizable patches of difference ED that were identified and refined as phosphate/sulfate anions ($\sim 60\%$ occupied). Both phosphate positions correlate well with phosphate groups of substrates or analogs that were found in the other RuBisCO structures (28) (Fig. 3A).

Activation. Subsequently, to investigate the active enzyme, we attempted to activate the *G. sulphuraria* RuBisCO in both soluble and crystal forms. We were unable to activate the enzyme using up to 20 mM Mg^{2+} and excess CO_2 . Nevertheless, when the crystals

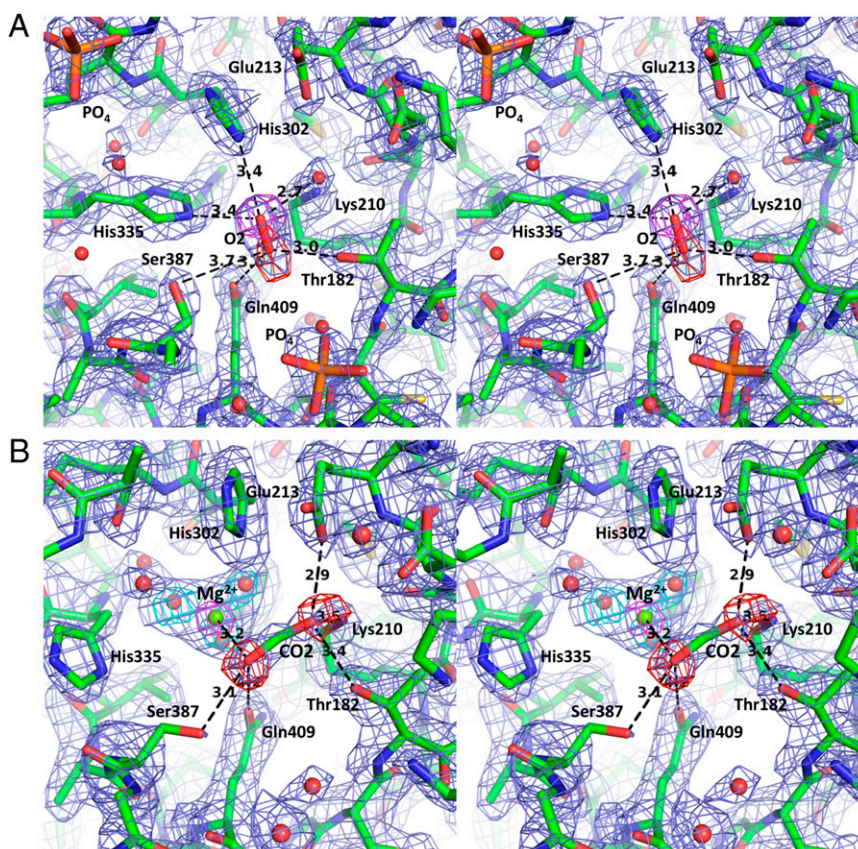


Fig. 3. The stereo representation of the active site of the enzyme (in bond-stick). (A) The apo-enzyme with O_2 bound at the C-terminal end of the inner β -barrel constituting the active site. The $2Fo-Fc$ electron density contoured at the 1σ level is in slate blue, whereas the two omit maps for individual oxygen atoms in the O_2 molecule are contoured at the 4σ level (purple and red). (B) The active site of the preactivation complex with Mg^{2+} surrounded by three water molecules and the CO_2 molecule. The $2Fo-Fc$ ED map (contoured at the 1σ level) is in slate blue; the omit maps, calculated with oxygen atoms omitted from the CO_2 molecule (in red), are contoured at 4σ ; the omit map for Mg^{2+} (in green) is contoured at the 6σ level; and the omit maps for the surrounding water molecules are contoured at 3.5σ (in cyan).

222 (30). The role of activation temperature associated with protein's mobility in increasing the level of specificity, as highlighted by the kinetics of RuBisCO (from *Thermococcus kodakarensis*) that exhibits S of ~ 310 at 90°C but only S of ~ 6 at 25°C (31), needs to be further examined.

The quadrupole moments of charge-less and dipole-less ligands interact only with the high electrostatic field gradient that provides a sufficient guiding force for discrimination and binding at the active site. This apparent need for a high gradient at the active site region explains why the minimal active form of RuBisCO is a dimer of large subunits. Unlike monomers, only dimers produce sufficiently strong electric field gradient across the active site by combining highly and oppositely charged domains that are required for the interaction of RuBisCO with the gaseous substrates. The dimeric form recombines the monomers that have negatively and positively charged elements separated into the N-terminal (dimerization) and the C-terminal (catalytic) domains to avoid misfolding. This conclusion is partially supported by the use of chaperones in the larger oligomer assembly (L8S8) (32).

This modular design of RuBisCO also suggests a function for many flexible loops present at the active site. During catalysis, the enzyme closes the C- and N-terminal segments (33) to increase the strength of the electric field by decreasing the dielectric constant and simultaneously sealing off the active site from solvent. Closing of the active site loops has a high entropic cost; therefore, it is rare and results in a low turnover rate. These conclusions agree well with claims that the specificity is mostly imparted by the mobile loops of the enzyme (16), but also agree with modest improvement in the catalytic efficiency and selectivity of RuBisCO achieved in the mutational studies (9, 15).

Mechanism of Carbamylation. The structures described above suggest the mechanism of RuBisCO activation by Lys210 carbamylation. Initially, the rotation of His335 to its alternative conformation opens up the active site and creates a transient binding site for the Mg^{2+} ion. When two water molecules bound by the Mg^{2+} ion are transiently converted to OH, which results in partial neutralization, the hydrated metal binds at the active site defined by three His (300, 302, 335). His residues (Fig. 3 and Fig. S6) coordinate the metal cation indirectly through the water molecules, which results in a weak and unstable complex. This environment favors binding of Mg^{2+} in a rare pseudotetrahedral coordination with three $\text{H}_2\text{O}/\text{OH}$ molecules and one CO_2 molecule bound at the solvent-exposed tetrahedral site. The metal-bound hydroxides drive the deprotonation of the Ne of Lys210 that results in a conformational change in which the Ne of Lys210 rotates by 120° to the *trans* conformer. This rotation significantly decreases the distance between the Ne of Lys and the C atom of CO_2 . Next, the CO_2 forms a covalent bond with the Ne atom of Lys210 and creates a negatively charged carbamyl group that facilitates metal-ion binding. As a result of CO_2 covalent attachment to Lys210, the destabilized Mg^{2+} ion relocates to a new binding site, created by the carbamyl group of Lys210 and the carboxyl groups of Glu-213 and Asp212. At the final step, His335 that undergoes thermal fluctuation returns to its original conformation, thus finalizing the activation and the formation of the mature active site (Fig. S6)

and thus enabling a full closure of loop 6 (Fig. S2) upon binding of the substrates.

The RuBisCO activation mechanism has a chemical precedent in CA. Its reverse reaction (to primary dehydration) mirrors the proposed mechanism (albeit resulting in a different product) (34). In the CA complex with CO_2 , the position of the activated water is similar to that of the alternative position of the Lys210 Ne, compared with a model of the activation mechanism (Fig. S6). The carbonic acid reaction of CA therefore provides a prototype for the carbamylation reaction in RuBisCO (Fig. S2 B and C).

Conclusions

In this paper, I propose the mechanism of activation of RuBisCO. This work provides an insight into the elusive preactivation complex with Mg^{2+} and CO_2 and defines the principles for the gaseous ligand differential binding. I conclude that the trapping of the intermediate and gaseous ligands becomes possible because the enzyme is inactivated by nitrosylation. I believe that nitrosylation is a common mechanism for controlling RuBisCO activity that has not received sufficient attention.

All of the factors described in this paper, i.e., existence of a strong electric field gradient that interacts with quadrupole moments of the substrates, temperature activation associated with protein mobility, and finally directional binding of the ligands, affect the efficiency of the enzyme, and thus they all should be considered in reengineering of the enzyme to improve its catalytic efficiency and the substrate specificity. The methods for proper accounting for changes in the electrostatic field interactions with the quadrupole moment of the ligands as well as the mobility factors in the mutant proteins must be first developed to fully use the findings of this report. In light of the results presented in this study, I propose that the dimeric forms of RuBisCO are much more amenable material for genetic manipulations and future successful reengineering of the enzyme for practical applications.

Materials and Methods

Protein production and crystallization methods have been described before (21). The native enzyme was inactive. The activation process was carried out by treating the enzyme with 20 mM MgSO_4 under CO_2 atmosphere for an extended period (up to 24 h). We did not manage to activate the purified enzyme. The activation process of the protein crystals was attempted at room temperature as well as at the elevated temperature ($\sim 40^\circ\text{C}$). The crystals were usually harvested after ~ 2 h in activating conditions. The crystals of the wild-type enzyme subjected to treatment with Mg^{2+} and CO_2 were immediately frozen in liquid nitrogen upon harvesting from the crystallization wells. The data were collected at low temperature (100 K) for the preactivation complexes whereas the data for the WT native crystals were collected at room temperature. The data were collected on the Rigaku Microfocus 007 and Raxis IV++ imaging plate detector. We refined the structures using Shelx93 and Refmac. The use of both programs was instrumental in identifying gaseous ligands. The ligands were identified by several criteria: (i) elimination of residual difference densities, (ii) comparable level of B-factors to surrounding atoms, and (iii) proper hydrogen-bonding patterns. Their identity was confirmed by omit maps. The refinements converged with excellent stereochemistry, and the final model fit to the ED maps. The R (Rfree) of the refined models was 0.166 (0.22) and 0.181 (0.241) for the native and preactivation complex with CO_2 and 0.163 (0.234) for the activation complex without gaseous ligands. For a summary of the refinement statistics, see Table S1.

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