The Unique Pentagonal Structure of an Archaeal Rubisco Is Essential for Its High Thermostability*

We have previously determined the crystal structure of a novel pentagonal ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) from the hyperthermophilic archaeon, Thermococcus kodakaraensis KOD1. Here we have carried out biochemical studies to identify the necessities and/or advantages of this intriguing pentagonal structure. The structure indicated the presence of three neighboring residues (Glu-63, Arg-66, and Asp-69), participating in ionic interactions within unique dimer-dimer interfaces. We constructed three single mutant proteins (E63S, R66S, and D69S) and one triple mutant protein (E63S/R66S/D69S) by replacing the charged residues with serine. The wild type (WT) and all mutant proteins were purified and subjected to gel permeation chromatography at various temperatures. WT and D69S proteins were decameric at all temperatures examined between 30 and 90 °C. The majority of E63S and R66S were decamers at 30 °C but were found to gradually disassemble with the elevation in temperature. E63S/R66S/D69S was found in a dimeric form even at 30 °C. An interesting correlation was found between the subunit assembly and thermostability of the proteins. Circular dichroism and differential scanning calorimetry analyses indicated that the denaturation temperatures of dimeric enzymes (E63S, R66S, and E63S/R66S/D69S) were ~95 °C, whereas those of the enzymes retaining a decameric structure (WT and D69S) were ~110 °C. Disassembly into tetramer or dimer units did not alter the slopes of the Arrhenius plots, indicating that the decameric structure had no effect on catalytic performance per se. The results indicate that the decameric assembly of Tk-Rubisco contributes to enhance the thermostability of the enzyme. Taking into account the growth temperature of strain KOD1 (65–100 °C), the decameric structure of Tk-Rubisco can be considered essential for the stable presence of the enzyme in the host cells. This study provides an interesting example in which the thermostability of a protein can be enhanced by formation of a unique quaternary structure not found in mesophilic enzymes.

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39) is one of the key enzymes in the Calvin-Benson cycle and is the most abundant enzyme on our planet (1–4). The carboxylase activity of this single enzyme is responsible for the carbon dioxide fixation in all plants, algae, and many bacteria, including cyanobacteria. Ribulose 1,5-bisphosphate, carbon dioxide, and water are converted to two molecules of 3-phosphoglycerate (3-PGA), which is eventually utilized for the production of all organic cell materials. Besides the carboxylase activity, Rubisco also displays a competing oxygenase activity. In the oxygenase reaction, one molecule of 3-PGA and one molecule of 2-phosphoglycolate are produced from ribulose 1,5-bisphosphate and molecular oxygen. The oxygenase activity of Rubisco actually decreases the net fixation of carbon dioxide in the organism, and the physiological meaning of this reaction is yet to be clarified (1, 2).

Until recently, Rubiscos had been classified into two types (or forms) based on their quaternary and primary structures (1–3). Type I Rubisco, found in higher plants, algae, cyanobacteria, and many other bacteria, is composed of eight large (L) subunits and eight small (S) subunits, forming a heterohexameric fold of the enzyme. The large and small subunits are encoded by two different genes and assemble into the large (L2S2) and small (S2S2) dimers, respectively. Type II Rubisco from some bacteria consists of large subunits only (L2). This classification, based on subunit composition, coincides well with the relatedness of amino acid sequences among the large subunits (4). Sequence similarity among large subunits of the same type is relatively high (>70%), whereas those among different types exhibit only 50% similarity. The large subunits of both types display a similar protein fold and comprise two domains, a small N-terminal domain and large C-terminal domain. A dimeric structure, containing two active sites, can be considered the minimal unit for Rubisco activity, as residues from both subunits contribute to form the active center (1, 2).

We have previously discovered that Rubiscos are also present in hyperthermophilic Archaea (5). The physiological roles of archaeal Rubiscos are still unknown, and their primary structures are distinct to those of the type I and II Rubiscos described above. We have carried out gene analysis (5), biochemical characterization (5–7), and three-dimensional structural analysis (8) of the Rubisco from Thermococcus kodakaraensis KOD1 (Tk-Rubisco). The enzyme harbored unprecedentedly high carboxylase activity and carboxylase specificity besides its extreme thermostability. Immunochemical studies indicated that the enzyme was composed solely of large subunits (7). The three-dimensional structure revealed that although the monomeric fold of the enzyme resembled those of type I and type II enzymes, the subunit assembly of the enzyme was unique.

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Tk-Rubisco proved to be an (L2)₅ decamer harboring one 5-fold and five 2-fold symmetry axes perpendicular to each other. Compared with previously reported type I enzymes, each dimer was inclined ~16° along its 2-fold symmetry axis, forming a toroid-shaped decamer. Therefore, the dimer-dimer interfaces found in Tk-Rubisco were distinct to those of previously known Rubiscos. These interfaces occupied only 3.3% of the solvent-accessible surface area of a dimer, but the structure suggested the presence of many ionic interactions within these interfaces (8). Tk-Rubisco possessed one extra α-helix (helix αO, residues 64–70), and three neighboring residues located in or near this helix (Glu-63, Arg-66, and Asp-69) were supposed to mainly participate in these ionic interactions between adjacent dimers (8).

In this study, we have constructed mutant proteins intending to disrupt the ionic interactions in the dimer-dimer interface of Tk-Rubisco. By characterizing these proteins, we addressed the following two subjects. First, we aimed to determine the residues and the interactions that contribute to the decameric structure of Tk-Rubisco. Second, we were interested in whether the decameric structure of Tk-Rubisco per se had any meaning in terms of the biochemical properties of the enzyme. Would a decameric structure have any advantages compared with a type II-like dimeric structure? As a result, this study provides clear evidence that the decameric structure of Tk-Rubisco, supported by intersubunit ionic interactions, is essential for the thermostability of the protein at the growth temperatures of its host, *T. kodakaraensis* KOD1.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Media—**Escherichia coli DH5α and the vector pUC18 were used for cloning and gene manipulation. For overexpression of the Tk-Rubisco gene (*Tk-rbcL*) and its mutant genes, *E. coli* BL21(DE3) (Stratagene, La Jolla, CA) and the vector pET21a (+) (Novagen, Madison, WI) were utilized. LB medium was used for cultivation of *E. coli* (9).

**Site-directed Mutagenesis—**Site-directed mutagenesis was performed using QuikChange site-directed mutagenesis kit (Stratagene). To substitute Glu-63, Arg-66, and Asp-69 with serine, sequences within the *Tk-rbcL* gene (accession number AB018555), namely GAG (bases 157–189), AGA (bases 196–198), and GAC (bases 205–207), were replaced with AGC, respectively. The sequences of the mutated genes were confirmed using the ABI PRISM kit and Model 310 capillary DNA sequencer (Applied Biosystems). The mutant genes were inserted into pET21a (+) after digestion with Nde1 and SalI.

**Gene Expression and Purification of Wild Type and Mutant Proteins—**After insertion of the *Tk-rbcL* and its mutant genes into pET21a (+), the plasmids were independently introduced into *E. coli* BL21(DE3)/CodonPlus. Gene expression was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h. After harvesting, the cells were suspended in 100 mM Bicine-KOH (pH 8.3), 10 mM MgCl₂ (Buffer A) and disrupted by sonication. The soluble cell-free extracts were heat-precipitated for 30 min at 85 °C. The supernatant was collected every 0.5 min and the temperature of the midpoint of the transition (Tₐ) was determined by monitoring the change in the CD value at 220 nm. Data were collected every 0.5 °C. Temperature elevation was carried out with a 0.5 °C elevation during a 30-s period, and after incubation at the temperature for 1 min, measurements were carried out. This corresponds to a 1 °C/min elevation rate.

**Activity Measurements—**Carboxylase activity was measured as described elsewhere (10). The enzyme solution contained 5 µg of enzyme in 80 µl of 100 mM Bicine-KOH (pH 8.3), 10 mM MgCl₂ buffer (Buffer A). A 10-µl aliquot of 1 mM NaHCO₃ in Buffer A was added. After incubation at the desired temperature for 3 min, a 10-µl aliquot of 300 mM ribulose 1,5-bisphosphate in Buffer A was added, and incubation was continued for another 3 min. After stopping the reaction, the solution was appropriately diluted (5–50 times), and 30 µl was added to a coupling mixture to quantify the 3-PGA formed. The coupling reaction contained 563 units/ml 3-phosphoglycerate phosphokinase, 125 units/ml glyceraldehyde-3-phosphate dehydrogenase, 260 units/ml triose-phosphate isomerase, and 22.5 units/ml glyceraldehyde dehydrogenase along with 0.8 mM NADH, 5 mM ATP, and 5 mM reduced glutathione in a 150 mM Bicine-KOH (pH 8.3), 15 mM MgCl₂ buffer. The difference in absorbance at 340 nm before and after the coupling reaction was measured. A linear correlation was detected between the decrease in absorbance and the amount of 3-PGA at concentrations between 3 and 150 µM.

**RESULTS**

**Extensive Ionic Interaction in the Dimer-Dimer Interface—**We have previously determined the first crystal structure of an archaeal Rubisco from the hyperthermophile, *T. kodakaraensis* KOD1, at 2.8-Å resolution (8). The protein fold of the monomer highly resembled those of previously determined Type I Rubiscos from spinach (11), tobacco (12), *Synechococcus* (13), *Galdieria partita* (14), and *Alcaligenes eutrophus* (15) along with the type II enzyme from *Rhodospirillum rubrum* (16). However, a significant structural difference was observed in its subunit assembly. Tk-Rubisco consisted of 10 large subunits and displayed a novel pentagonal structure with unique dimer-dimer (L₂-×₂) interfaces (8). When 3.2 Å was applied as the cut-off criterion, 10 residues were presumed to be involved in dimer-dimer interactions, forming 1 hydrogen bond and 8 ionic bonds. The structure suggested that Glu-63, Arg-66, and Asp-69 were involved in 6 of these ionic interactions (Fig. 1, A and B). These residues are located in αII or adjacent to an extra α-helix (helix αO), present in Tk-Rubisco, spanning the region Gln-64 to Leu-70. Therefore, it was assumed that these charged residues were involved in maintaining the decameric structure of the enzyme. Thus, we constructed the following three single mutants and one triple mutant and investigated the properties of each mutant protein. The three single mutants, in which Glu-63, Arg-66, or Asp-69 was replaced with a serine residue were designated as E63S, R66S, and D69S, respectively. The triple mutant, in which all three charged residues were replaced with Ser, was named E63S/R66S/D69S.

**Gene Expression and Purification of Wild Type and Mutant Rubisco Proteins—**Wild type and mutant Rubisco genes were expressed in *E. coli*, and the mutant proteins were retained in a soluble form. The cell-free extracts were heat-precipitated for 30 min at 85 °C, as carried out for the wild type enzyme. All mutant proteins were stable against this treatment and remained in their soluble forms. Anion exchange chromatography and gel filtration chromatography were performed to purify the proteins. In the anion exchange chromatography, the
mutant D69S protein was eluted at the same salt concentration (−0.3 M NaCl) as the wild type protein, but the triple mutant protein was eluted at a higher salt concentration (−0.4 M NaCl). Interestingly, E63S and R66S proteins were detected in two separated fractions, eluting at −0.3 M and 0.4 M NaCl concentrations (data not shown). This correlation was also observed with gel filtration chromatography. The molecular mass of D69S was equivalent to that of the wild type protein, indicating a decameric structure. In contrast, the elution profile of E63S/R66S/D69S suggested that the triple mutant protein was a protein with lower molecular mass. E63S and R66S proteins were predominantly decamers, but a small population of these proteins was also found in the lower molecular mass fractions (data not shown). We have previously reported that an extensive number of acidic residues occupy the inner surface of the pentagonal ring of Tk-Rubisco (8). A decameric structure maintains this region on the inner side of the enzyme, but disassembly of the decamer would allow the acidic surface to interact with the anion exchange matrix. Therefore, elution of the triple mutant at 0.4 M NaCl concentration, elution of the wild type enzyme at 0.3 M, and elution of the E63S and R66S mutants at both 0.4 and 0.3 M coincide well with the results of gel filtration chromatography. When the decameric fractions were repeatedly applied to gel filtration chromatography, a small portion of the enzyme was detected in the lower molecular mass fraction, suggesting an equilibrium between these two states (see below). Decameric fractions of E63S and R66S were utilized for further experiments. After gel filtration chromatography, the purity of mutant proteins was confirmed by SDS-PAGE (data not shown).

The mutant proteins remained soluble after the heat treatment mentioned above, indicating that no drastic alteration in structure was introduced by the mutations. We further examined the effect of mutations on the secondary structure of each protein. Far-UV CD spectra of the mutant proteins were measured at 20 °C and compared with that of the wild type protein. The CD spectra of all proteins were virtually identical, suggesting that the mutations at the positions of 63, 66, and 69 (helix eO) did not induce major structural alterations (data not shown).

**Subunit Composition of the Mutant Proteins at Various Temperatures**—We next investigated the subunit composition of

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**Fig. 1.** A, unique dimer-dimer interface of Tk-Rubisco. The open oval indicates the dimer-dimer interface, where extensive ionic interactions are located. The closed oval in the center indicates the position of 2-fold symmetry axis perpendicular to the figure surface. B, schematic model of the extensive ionic interactions within the dimer-dimer interface of Tk-Rubisco.
mutant proteins at various temperatures using gel permeation chromatography. All proteins were applied at a concentration of 1 mg/ml. Because denaturation of protein molecular mass marker at high temperatures was expected, various chain lengths of pullulan were utilized as molecular mass markers. The elution profiles of wild type, E63S, R66S, and D69S, and E63S/R66S/D69S at 30 °C are shown in Fig. 2A. At this temperature, the profiles of wild type, E63S, and D69S proteins were indistinguishable, eluting at a retention time corresponding to a decameric protein. The profile of the R66S protein displayed an additional shoulder peak corresponding to a molecular mass of ~180 kDa or a tetrameric structure. In contrast, we found that
The E63S/R66S/D69S triple mutant eluted as a single peak, corresponding to a dimer. We could also observe a concentration-dependent transition between decamers and dimers when we carried out gel filtration chromatography with a Superdex 200 HR 10/30 column. In the case of the E63S mutant protein, a decamer-dimer ratio of 61:39 was observed at an initial protein concentration of 11.6 mg/ml, whereas the ratio was 14:86 at 0.48 mg/ml (data not shown).

To determine the stability of the decameric structure of each protein, gel permeation chromatography was then performed at elevated temperatures (Fig. 2B). The wild type enzyme retained a decameric structure at 30, 60, 80, and 90 °C (data not shown). The D69S mutant protein was also found to maintain a decameric structure even at 90 °C. The decameric structure of the E63S protein, which was found at 30 °C, gradually dissociated with the elevation in temperature. The majority of the protein was found to be a tetramer at 80 °C. As for the R66S protein, the majority was tetrameric at 60 °C and further dissociated into dimers at 80 °C. Experiments at 90 °C for the E63S and R66S proteins were not possible because denaturation of these proteins was observed (see below). These results strongly indicated that Glu-63 and Arg-66, particularly Arg-66, were involved in maintaining the decameric structure of Tk-Rubisco.

**Thermal Stability of Mutant Proteins**—The loss of the far-UV CD value at 220 nm was used to monitor the temperature dependence of thermal denaturation for each protein (Fig. 3). In the cases of E63S, R66S, and the triple mutant protein, the proteins were completely denatured at temperatures below 100 °C. After normalizing the data, the melting temperatures (T_m) or the midpoint of denaturation of E63S, R66S, and the triple mutant protein were 92.8, 90.8, and 89.6 °C, respectively. In contrast, the wild type protein was stable, and denaturation was not detected even at 100 °C.

Disruption of the D69S secondary structure initiates at temperatures above 90 °C, but the protein was not completely denatured at 100 °C.

Differential scanning calorimetry experiments were also carried out to determine the irreversible denaturation temperature of each protein. The results revealed an interesting correlation between the subunit assembly and thermostability of the proteins. Wild type and D69S proteins, which maintained decameric structures at 90 °C, were found to denature at 113 and 109 °C, respectively (data not shown). In contrast, the Tk-Rubisco mutant proteins that disassembled to dimers or tetramers at 80 °C (E63S, R66S, E63S/R66S/D69S) shared similar denaturation temperatures of 95, 94, and 94 °C, respectively (data not shown). Because it is likely that the E63S mutant protein is in a dimeric form at 90 °C, it can be estimated that the disassembly of decameric Tk-Rubisco into dimers lowers the denaturation temperature by −20 °C.

**Carboxylase Activity**—To compare the enzymatic properties of mutant proteins, the carboxylase activity of each protein was measured at various temperatures (Fig. 4A). Measurements were carried out by quantifying the 3-PGA formed by Tk-Rubisco with a coupled reaction including 3-phosphoglycerate phosphokinase, glyceraldehyde-3-phosphate dehydrogenase, triose-phosphate isomerase, and glycerophosphate dehydrogenase (see “Experimental Procedures” and Ref. 10). At all temperatures examined, wild type Rubisco showed the highest carboxylase activity among all enzymes. The carboxylase activity of wild type enzyme increased with increasing temperature, and the enzyme showed the highest carboxylase activity, 55.6 μmol of CO₂ fixed/min/mg of protein, at 100 °C. As for the mutant proteins, activities were in the order of D69S, E63S, R66S, and E63S/R66S/D69S. The activities of R66S and the triple mutant protein were extremely low and were less than 1.6 μmol of CO₂ fixed/min/mg protein even at their optimal temperatures of 80 °C (Fig. 4A). Similar tendencies were observed when activity measurements were performed by directly
quantifying 3-PGA with high performance liquid chromatography (data not shown).

Arrhenius plots of WT, D69S, and E63S were constructed to investigate the relationship between temperature and activity (Fig. 4B). An Arrhenius plot of wild type Tk-Rubisco was linear throughout a temperature range of 50–100 °C, whereas linearity was observed between 50 and 90 °C for D69S and E63S. From these results, it is likely that drastic conformational changes do not occur in these three proteins between 50 and 90 °C (50–100 °C in the case of the wild type protein). The drastic decrease in activity of E63S at 100 °C indicates that the protein begins to denature at temperatures between 90 and 100 °C. The same tendency, but to a lesser extent, was also observed for D69S. These results agree well with the results of Fig. 3. Moreover, the Arrhenius plot of E63S revealed that there was no significant relationship between subunit assembly and enzymatic activity. The results of gel permeation chromatography at various temperatures clearly indicated the disassembly of E63S from a decameric structure to a tetrameric structure within the temperature range of 30–80 °C. However, the slope of the Arrhenius plot of E63S was continuously linear throughout this temperature range, indicating that the pentagonal assembly of dimers per se had no effect on the catalytic performance of E63S.

**DISCUSSION**

Rubisco from *T. kodakaraensis* KOD1, Tk-Rubisco, displays an unprecedented pentagonal decameric structure, (L$_2$)$_5$, and three-dimensional structural analysis of the enzyme suggested the presence of extensive ionic interactions within the interfaces of adjacent dimers (8). The results of this study have clarified the importance of the ionic interactions in the maintenance of the pentagonal structure as well as identified the residues involved. Moreover, we were able to elucidate the necessity of the peculiar pentagonal structure of Tk-Rubisco.

The four mutant proteins, E63S, R66S, D69S, and E63S/R66S/D69S, were all produced as soluble proteins, displayed similar CD spectra, and were resistant to heat treatment at 85 °C for 30 min, indicating that the introduction of mutations did not drastically alter the fold of the proteins. This was strongly supported by the fact that all mutant proteins harbored Rubisco activity. Nevertheless, each mutant protein exhibited distinct properties in terms of subunit composition, thermostability, and activity levels. Wild type Tk-Rubisco can be presumed to maintain its decameric structure until the protein is denatured at −113 °C. The triple mutant protein was a dimer at all temperatures examined. It was less stable than the wild type protein, and denaturation was observed at −90 °C. As for the single mutant proteins, the majority of E63S and R66S proteins were decamers at 30 °C. The decameric structures of the two proteins dissociated to tetramers or dimers at elevated temperatures, and their denaturing temperatures were similar to the triple mutant protein (−90 °C). The results indicate that the dimeric structure of Tk-Rubisco itself denatures at −90 °C. This is also supported by the fact that the only mutant to maintain a decameric structure at 90 °C, D69S, displayed a denaturation temperature of 109 °C, comparable with the 113 °C of the wild type protein. Our results agree well with one another and commonly indicate that the pentagonal assembly of the Tk-Rubisco dimer units leads to an enhancement in thermostability.

Concerning the carboxylase activity of the proteins, wild type Tk-Rubisco showed the highest activity among all enzymes examined. Activities were in the order of WT > D69S ≈ E63S >> R66S ≈ E63S/R66S/D69S. Although this order of activity coincided with the order of thermostability, or higher oligomerization states, it is unlikely that the activity level is strongly influenced by the oligomeric state of the protein itself. There is a large difference in activity between wild type and D69S mutant proteins from 70 to 90 °C, although both are decamers in this temperature range. Similarly, D69S and E63S display similar levels of activity from 60 to 80 °C, despite their different oligomeric states. The strongest evidence is provided by the Arrhenius plot of the E63S protein. Although the oligomeric state of E63S changes between 30 and 80 °C, the slope of the Arrhenius plot is constant throughout this temperature range. Activity levels seem to be influenced by alterations within the dimer and not by dimer-dimer interactions. This may be due to a local conformational change of an active site residue Thr-54 in the immediate vicinity of helix αO, where the mutations were introduced.

Putative Rubisco and Rubisco-like orthologues have been identified on the complete genome sequences of numerous archaeal strains. Among these, the MJ1235 gene from *Methanococcus jannaschii* and the AF1638 gene from *Archeoglobus fulgidus* have been shown to encode proteins with *bona fide* Rubisco activity (17). The unique structure of Tk-Rubisco tempted us to search for the residues that participate in decamerization in other archaeal Rubisco sequences. The Rubisco from *M. jannaschii* has been reported to be a dimeric protein (17), and accordingly, conservation of the residues was very low, with only two presumable ionic interactions. Only three to four of the eight ionic interactions were conserved in the Rubiscos from *A. fulgidus* (AAB89603), *Pyrococcus furiosus* (AAL81280), *Pyrococcus abyssi* (CAB50122), and *Pyrococcus horikoshii* (BAAS0036). Although none of these proteins have been subject to three-dimensional structural analysis, sequence comparison does not support the possibilities that archaeal Rubiscos are decamers in general. These Rubiscos may harbor high thermostability by strengthening and/or increasing atomic interactions within their respective dimers.

The structural features that bring about the high thermostability of proteins from hyperthermophiles have attracted much attention. Structural comparison among protein counterparts from mesophiles, thermophiles, and hyperthermophiles has indicated some of the factors that lead to thermostability (18). A well studied example is triose-phosphate isomerase (19–21), and it has been found that the thermostable triose-phosphate isomerase proteins have severe pruning of some helices along with truncation of some loop structures, leading to a more compact monomer than its mesophilic counterparts. A higher oligomerization state of the thermostable protein has also been suggested to contribute to extreme thermostability (21). A few other enzymes from hyperthermophiles have also been reported to take a higher oligomerization state than those of their mesophilic counterparts (22). These include dihydrofolate reductase (23) and phosphoribosylanthranilate isomerase (24) from *Thermotoga maritima* and methenyltetrahydromethanopterin cyclohydrolase from *Methanopyrus kandleri* (25). The possibilities that the higher oligomerization states of these proteins contribute to enhance their thermostability have been discussed. The only study that has experimentally addressed this possibility was carried out with the phosphoribosylanthranilate isomerase from *T. maritima* (26). The results indicated that dimerization through hydrophobic interactions did indeed enhance the thermostability of phosphoribosylanthranilate isomerase from *T. maritima*. The present study on Tk-Rubisco provides a clear case where a unique quaternary structure of an enzyme from a hyperthermophile contributes to stabilize the protein from thermal inactivation. Decamerization increased the denaturation temperature of the protein from −90 °C to 113 °C. Taking into account that the growth temperature of *T. kodakaraensis* KOD1 ranges from 65 to 100 °C (27),
the decameric structure of Tk-Rubisco can be considered essential for the stable presence of the enzyme in its native host cell.

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