The sequences classified as genes for various ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (RuBisCO)-like proteins (RLPs) are widely distributed among bacteria, archaea, and eukaryota. In the phylogenetic tree constructed with these sequences, RuBisCOS and RLPs are grouped into four separate clades, forms I–IV. In RuBisCO enzymes encoded by form I, II, and III sequences, 19 conserved amino acid residues are essential for CO2 fixation; however, 1–11 of these 19 residues are substituted with other amino acids in form IV RLPs. Among form IV RLPs, the only enzymatic activity detected to date is a 2,3-diketo-5-methylthiopentyl 1-phosphate (DK-MTP-1-P) enolase reaction catalyzed by Bacillus subtilis, Microcystis aeruginosa, and Geobacillus kaustophilus form IV RLPs. RLPs from Rhodospirillum rubrum, Rhodopseudomonas palustris, Chlorobium tepidum, and Bordetella bronchiseptica were inactive in the enolase reaction. DK-MTP-1-P enolase activity of B. subtilis RLP required Mg2+ for catalysis and, like RuBisCO, was stimulated by CO2. Four residues that are essential for the enolization reaction of RuBisCO, Lys175, Lys201, Asp203, and Glu204, were conserved in RLPs and were essential for DK-MTP-1-P enolase catalysis. Lys233, the residue conserved in DK-MTP-1-P enolases, was also essential for B. subtilis RLP enolase activity. Similarities between the active site structures of RuBisCO and B. subtilis RLP were examined by analyzing the effects of structural analogs of RuBP on DK-MTP-1-P enolase activity. A transition state analog for the RuBP carboxylation of RuBisCO was a competitive inhibitor in the DK-MTP-1-P enolase reaction with a Ki value of 103 μM. RuBP and d-phosphoglyceric acid, the substrate and product, respectively, of RuBisCO, were weaker competitive inhibitors. These results suggest that the amino acid residues utilized in the B. subtilis RLP enolase reaction are the same as those utilized in the RuBisCO RuBP enolization reaction.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) catalyzes the carboxylation and oxygenation reactions of ribulose-1,5-bisphosphate (RuBP) in photosynthesis (1–4). This enzyme is the sole CO2-fixing enzyme in plants; however, it has certain inefficiencies. It has a very low turnover rate, a low affinity for the substrate, CO2, and low specificity between the carboxylation and oxygenation reactions (5–7). Thus, the intrinsic enzymatic properties of RuBisCO are inadequate for efficient incorporation of CO2 into organic matter in photosynthesis (7). However, plants have overcome these disadvantages by investing a huge amount of leaf nitrogen in RuBisCO synthesis (8).

In nature, there are wide variations in the properties and primary sequences of RuBisCO among different photosynthetic organisms (9–12). The primary sequences vary as much as 73% without loss of activity. The relative specificity ranges from ~0.5 in a small subunitless RuBisCO to 238 in a red algal, hexadecameric RuBisCO (13, 14). The affinity for CO2 varies some 100-fold (15). Comparisons between these kinetic parameters and the primary sequences are expected to reveal promising strategies for improving the enzyme, and many studies have been conducted on this topic (7, 16–18).

A RuBisCO-like protein (RLP) with no CO2-fixing activity was first demonstrated in Chlorobium tepidum (19), and a similar protein in Bacillus subtilis was found to be involved in the methionine salvage pathway (20). These findings have pointed to a new direction in RuBisCO research (17, 21). The phylogenetic tree of the catalytic subunits of RuBisCOs and their homologs shows four major clusters, forms I–III, and form IV (Fig. 1A). Form I and II RuBisCOS are involved in photosynthetic or chemosynthetic CO2 fixation, whereas the metabolic function of form III RuBisCOS remains unclear, although they can fix CO2 on RuBP (9, 22). Forms I–III conserve almost all 19 amino acid residues that are essential for CO2 fixation in RuBisCO (Fig. 1B). The form IV cluster in the phylogenetic tree consists of RLPs that show ~20% homology to plant form I or bacterial form II RuBisCOS (12, 20, 21, 23–25). There are 8–18
RuBisCO-essential residues that are conserved in RLPs (Fig. 1B). Form IV RLPs are further subdivided into four groups; α1, α2, β, and γ (21). The RLP of *B. subtilis* is classified in α1 and catalyzes the enolization reaction of 2,3-diketo-5-methylthiopentyl 1-phosphate (DK-MTP-1-P) but not the carboxylation of RuBP (Fig. 2A) (20, 21, 23). The absence of CO₂-fixing activity in the *B. subtilis* RLP may be ascribed to changes in 8 of the 19 amino acid residues essential for CO₂ fixation in RuBisCO (Fig. 1B). Several of these residues are located at the C-terminal domains of *B. subtilis* RLP and RuBisCO. The dimeric RuBisCO from *Rhodospirillum rubrum* catalyzes the DK-MTP-1-P enolase reaction with very low activity (20). These findings, together with the similarity in the chemical structures of substrates for *B. subtilis* RLP and RuBisCO (Fig. 2A), suggest that they may have a close evolutionary relationship (12, 21, 23–25).

The RuBisCO reaction starts with the abstraction of the C3 proton from RuBP to form the cis-enediol (ate) of RuBP (Fig. 2A) (26). Using the spinach numbering format to identify RuBisCO and RLP residues, the carbamate formed on the ε-amino group of Lys201 may be the general base to abstract the proton, and the cis-enediol (ate) form of RuBP is stabilized in the combination of side chains from Lys175 and His294 (27). Asp203, Glu204, and the carbamylate Lys201 of the enzyme active site stabilize the cis-enediol (ate) and CO₂ through the Mg²⁺ ion (26). The *B. subtilis* RLP abstracts the C1 proton of its substrate DK-MTP-1-P to start the DK-MTP-1-P enolization reaction (12, 21, 23). The ε-amino group of Lys123 is thought to be required for the abstraction of the 1-proS proton in the *Geobacillus kaustophilus* RLP, which belongs to group α1, together with the *B. subtilis* RLP (Fig. 2B) (25). Lys123 is conserved among DK-MTP-1-P enolases and resides very near the C1 of 2,3-diketoheptane 1-phosphate (DK-H-1-P), a structural analogue of DK-MTP-1-P. As is the case in RuBisCO, the enolate intermediate is stabilized by Mg²⁺ and several amino acid residues: Lys175, Asp203, Glu204, His294, and the carbamylated Lys201.

The results of these studies suggest that the DK-MTP-1-P enolase is structurally and functionally related to photosynthetic RuBisCO. However, research on the *G. kaustophilus* RLP revealed that the proton-abstracting, reaction-starting residues differed between the DK-MTP-1-P enolase and RuBisCO (25). It has been reported that when lysine at 201 is substituted with an alanine in the *G. kaustophilus* RLP, the enzyme is still capable of catalyzing enolization of DK-MTP-1-P (25). This result raises a question about the above hypothesis on the close evolutionary relationship between the RLP and RuBisCO, because a carbamylated lysine residue would be required at this position to form the Mg²⁺-chelating triad linkage together with Asp203 and Glu204 and to stabilize the reaction intermediate in the RuBP enolization reaction of RuBisCO.

Evolutionary relationships of genes with similar sequences are deduced by comparing gene sequence homology of the genes and amino acid sequence homology of the predicted proteins and by analyzing conservation of functional motifs of the predicted proteins in silico. Comparison of protein structures at the active sites also provides important information. However, it may difficult to predict their mutual evolutionary relationship more precisely when they catalyze different reactions in individual metabolic pathways. The present research adopted a new method to resolve such an issue.

We studied the structural and functional interrelationships of RLP and RuBisCO after enzymological characterization of *B. subtilis* RLP as the DK-MTP-1-P enolase enzyme. The results showed that DK-MTP-1-P enolase activity was limited to some RLPs in the cluster, including *B. subtilis* in form IV RLPs. All of the catalytic residues for the RuBisCO reaction were also indispensable for DK-MTP-1-P enolase activity. The architecture of the *B. subtilis* RLP substrate-binding residues stereospecifically stabilized the transition state analog in CO₂ fixation of RuBisCO. The fact that the transition state analog of RuBisCO interacts with the active site of *Bacillus* RLP strongly supports their evolutionary proximity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Xylulose 1,5-bisphosphate (XuBP) was synthesized from dihydroxyacetone phosphate and glycol aldehyde phosphate by the method of Yokota (29). The product was separated on a Super-Q Toyopearl column (Toso) and eluted with a linear gradient of LiCl. XuBP fractions were pooled. The concentration of XuBP was measured as reported elsewhere (29).

The racemic mixture of 2-carboxy-d-arabinitol 1,5-bisphosphate (CABP) and 2-carboxy-d-ribitol 1,5-bisphosphate (CRBP) was synthesized by the method of Pierce et al. (30). Enantiomers were separated from each other by ionic chromatography on a MonoQ 5/50 column (GE Healthcare) and eluted with a 0–0.4 M linear gradient of LiCl in 3 mM HCl. Fractions with peaks in UV absorbance at 215 nm were assayed for total phosphate (31). The phosphorous peak fractions were separately pooled, and Li⁺ was replaced with Na⁺ using a Dowex 50 column (Na⁺ form). Concentrations of CABP and CRBP were calculated from their phosphate content. Other chemicals were analytical grade and were obtained from commercial sources.

**Preparation of Recombinant RLPs**—*R. rubrum* (National Institute of Technology and Evaluation Biological Resource Center number 3986) was purchased from the National Institute of Technology and Evaluation (Tsukuba, Japan). *R. rubrum* was cultured in 702 medium (pH 7.0) containing 10 g/liter polypeptone, 2 g/liter yeast extract, and 1 g/liter MgSO₄·7H₂O at 30 °C. Genomic DNA of *R. rubrum* was extracted with a DNeasy tissue kit (Qiagen). Genomic DNA from *G. kaustophilus tepidum* was kindly provided by Professor Hirozo Oh-oka (Osaka University). The gene for the *Microcystis aeruginosa* PCC 7806 RLP was obtained from Dr. N. Tandeau de Marsac (Pasteur Institute) (24).

The primers for PCR were as follows: CATCATATGACGGACAGACTGCCG and ACCGATCCCTTTGGCGACCTT-GAC for *R. rubrum*; GACGGGAATCACAATGATGTGA-GAAGACG and CCAGCCTGACTTTTTAGTCTGTC for *C. tepidum*; and CAGGTTGTCCATAGCTAATTGCACATTTG and CAAATTTGGCGGATCAGACTCAC for *M. aeruginosa*. Restriction sites for NdeI and BamHI are underlined. The gene for the *B. subtilis* RLP was cloned into pDG148 plasmid (32) using an upstream primer introducing a Sall site (underlined), ACCGTGCGACGCGATTGCGATTGAGAG.
and a downstream primer introducing an SpI site (underlined), ACATGCATGCAGGTGGTTCGAGTCGTCATACG.

Genes for RLPs of Rhodopseudomonas palustris CGA009 rlpl2 (IV-2 in Fig. 1) and Bordetella bronchiseptica RB50 were purchased from GenScript Corp. These genes were synthesized so that codon usage was optimized for Escherichia coli and were flanked by NdeI and BamHI sites. The DNA sequences for RLPs were cloned into pET15b vectors (Novagen, Tokyo, Japan). The pET15b-RLP genes were transformed into E. coli BL21 (DE3) (Novagen).

Site-directed Mutagenesis—The gene for the B. subtilis RLP was mutagenized in a site-directed manner to give K123N, K123E, K175N, K175E, K201N, and K201E using a QuikChange XL site-directed mutagenesis kit (Stratagene, Paris, France). The mutagenic primer sets used for PCR mutagenesis are listed in Table S1. The thermocycling program was as follows: denaturation for 1 min at 95 °C, followed by 18 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and elongation at 68 °C for 14 min, and termination with an additional elongation for 7 min.

Other mutations, K123A, K175A, K201A, D203N, D203E, E204Q, and E204D, were introduced using mutagenic primer sets (Table S1) with the following thermocycling program: 30 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and elongation at 74 °C for 30 min. Successful mutagenesis was confirmed by sequencing. The mutated B. subtilis RLP genes were also transformed into E. coli BL21 (DE3) with pET15b.

Expression and Purification of Recombinant RLPs and Mutated Proteins—Recombinant RLPs and mutated proteins were expressed and purified as reported previously (33, 34).

E. coli BL21 (DE3) was used for expression of recombinant RLPs and mutated B. subtilis RLPs. Histidine-tagged RLPs were purified with His-Bind resin (Novagen) according to the manufacturer's instructions. After purification, the RLP fraction was passed through a PD-10 column (GE Healthcare) equilibrated with 50 mM Tris-HCl buffer (pH 8.2) containing 150 mM NaCl, 2.5 mM CaCl$_2$, and 10% (v/v) glycerol. The active fractions were collected. The His$_6$ tag was excised with 2.6 U of thrombin/ml of recombinant protein for 3 h at 22 °C. Recombinant RLPs and mutated B. subtilis RLPs were further purified on a Superose 6 10/300 GL column (GE Healthcare) equilibrated with 50 mM Tris-HCl buffer (pH 8.2) containing 150 mM NaCl, 1 mM MgCl$_2$, and 10% (v/v) glycerol. The active fractions were pooled and stored at −80 °C until use. Native PAGE and SDS-PAGE were carried out as reported previously (35, 36).

Assay of DK-MTP-1-P Enolase—DK-MTP-1-P enolase activity was assayed in the coupling reaction with methyliothiobulose-1-phosphate (MTRu-1-P) dehydratase (20, 24, 33, 34). The substrate and the coupling enzyme were prepared as described previously (35). The reaction was initiated by adding 1.3 μg of methyliothiobulose-1-phosphate dehydratase to a mixture containing 50 mM Tris-HCl buffer (pH 8.2), 1 mM MgCl$_2$, 2 mM MTRu-1-P, and 0.9 μg of DK-MTP-1-P enolase. The final reaction volume was 100 μl and the reaction temperature was 35 °C unless otherwise stated. The concentration of the reaction substrate, DK-MTP-1-P, was calculated on the basis of the reaction characteristics between 2,3-butanedione and o-phenylenediamine (37, 38). The concentration of the reaction product, 2-hydroxy-3-keto-5-methylthiopentenyl 1-phosphate was monitored spectrophotometrically at 280 nm (20, 24, 33, 34). Enolase activity was calculated from the absorbance change within the initial seconds of the reaction. Within this time, the consumption of substrate, estimated based on the amount of the product, was less than 10%.

Changing of the CO$_2$ concentration in the reaction mixture, where specified, was done by removal of dissolved inorganic carbon, as reported by Matsuda and Colman (39). A mixture of DK-MTP-1-P enolase (0.1 mg/ml) and methyliothiobulose dehydratase (2 mg/ml) was added to a column (0.7 × 26 cm) of Sephadex G-25 (GE Healthcare) equilibrated with 50 mM Tris-HCl buffer (pH 8.2) containing 1 mM MgCl$_2$, which had previously been equilibrated with N$_2$ (N$_2$ > 99.99%) (Iwatani) for 30 min at 100 °C. The exact concentration of CO$_2$ in the reaction mixture after adding CO$_2$-free MTRu-1-P was calculated from the concentration of the dissolved inorganic carbon that was measured with a CO$_2$ analyzer (LI-6252; LI-COR, Lincoln, NE) (40).

RESULTS

Distribution of DK-MTP-1-P Enolase Activity among RLPs—Although some bacteria, archaea, and eukaryota have been reported to possess RLP genes, the only known function of these RLPs is enolization of DK-MTP-1-P to 2-hydroxy-3-keto-5-methylthiopentenyl 1-phosphate in RLPs from B. subtilis, G. kaustophilus, and M. aeruginosa classified in group α1 (20, 24, 25). However, there is no information regarding the distribution of this function among other RLPs in other groups or regarding functional analysis of the amino acid residues that may be involved in the DK-MTP-1-P enolization reaction.

To this end, we compared DK-MTP-1-P enolization activity (Table 1) and conservation among 19 amino acid residues in different RLPs and RuBisCOs. The activity with wild type normalized to 100%, which corresponded to 101.6 μmol min$^{-1}$ mg of protein$^{-1}$.

### TABLE 1

| Origin       | Group* | Relative activity* % |
|--------------|--------|----------------------|
| B. subtilis  | α1     | 100                  |
| M. aeruginosa| α1     | 39.1                 |
| R. rubrum IV | α2     | <0.01                |
| C. tepidum   | γ      | <0.01                |
| R. palustris IV-2 | β | <0.01                |

* Table 1 shows the distribution of DK-MTP-1-P enolase activity among RLPs and RuBisCOs. Enolase activity was calculated from the absorbance change within the initial seconds of the reaction. Within this time, the consumption of substrate, estimated based on the amount of the product, was less than 10%.

### RESULTS

Distribution of DK-MTP-1-P Enolase Activity among RLPs—Although some bacteria, archaea, and eukaryota have been reported to possess RLP genes, the only known function of these RLPs is enolization of DK-MTP-1-P to 2-hydroxy-3-keto-5-methylthiopentenyl 1-phosphate in RLPs from B. subtilis, G. kaustophilus, and M. aeruginosa classified in group α1 (20, 24, 25). However, there is no information regarding the distribution of this function among other RLPs in other groups or regarding functional analysis of the amino acid residues that may be involved in the DK-MTP-1-P enolization reaction.

To this end, we compared DK-MTP-1-P enolization activity (Table 1) and conservation among 19 amino acid residues in form IV RLPs (Fig. 1B). Activity was detected in RLPs from B. subtilis and M. aeruginosa in group α1, as reported elsewhere (20, 25). However, enolase activity was not detectable in several other form IV RLPs, including those of R. rubrum from group α2, B. bronchiseptica from group β, and R. palustris IV-2 and C. tepidum from group γ.

Of the 19 amino acid residues required for the RuBisCO reaction, Lys$^{175}$, Gly$^{196}$, Asp$^{198}$, Lys$^{201}$, Asp$^{203}$, Gly$^{204}$, His$^{294}$, Ser$^{297}$, Gly$^{381}$, Gly$^{383}$, and Gly$^{384}$ were conserved both in RuBisCOs and in RLPs with enolase activity. Gly$^{60}$, Lys$^{123}$, and Pro$^{295}$ were conserved in all RLPs with enolase activity, but these residues are Gly$^{60}$, Asn$^{123}$, and Asp$^{295}$ in RuBisCOs, respectively. Except for Arg$^{295}$, which is conserved in R. rubrum IV, these Gly$^{60}$ and Asn$^{123}$ residues are not conserved in form IV RLPs that show no enolase activity (Fig. 1B and Table 1).
**Similarities between Bacillus RLP and RuBisCO**

Mutational Analysis of the B. subtilis DK-MTP-1-P Enolase—
The RuBP-carboxylation reaction of RuBisCO is composed of five partial reactions: enolization of RuBP, carboxylation of the C2 carbon, hydration of the reaction intermediate, cleavage of the bond between the C2 and C3 carbons, and protonation of the aci-acid form of 3-phosphoglycerate (PGA) (Fig. 2A) (26). The enolization of RuBP is initiated with the abstraction of the C3 proton of RuBP by carboxamylated Lys\textsuperscript{201} together with Lys\textsuperscript{175}, and its cis-enediolate is formed between C2 and C3 of RuBP. On the other hand, the C1 proton of DK-MTP-1-P, a structural analogue of RuBP, is abstracted, and the enediolate is formed between C1 and C2 carbons in the DK-MTP-1-P enolase reaction (20). Binding and/or catalysis for RuBP enolization of RuBisCO is associated with four essential residues: Lys\textsuperscript{175}, Lys\textsuperscript{201}, Asp\textsuperscript{203}, and Glu\textsuperscript{204}. These are completely conserved among DK-MTP-1-P enolases (Fig. 1B). The importance of these residues has been shown by a structural comparison between the CABP-bound Spinacia oleracea RuBisCO and the G. kaustophilus RLP, which was reconstructed assuming its binding of DK-MTP-1-P in place of the reported compound DK-H-1-P (Fig. 2B) (25, 41).

These four essential residues and the Lys\textsuperscript{223} that is specific to the DK-MTP-1-P enolase were mutated to other amino acids to evaluate their functional significance for B. subtilis DK-MTP-1-P enolase activity. All mutant proteins were expressed in E. coli as soluble proteins and were purified in the same way as the wild-type recombinant protein (Fig. S1A). Native PAGE (Fig. S1B) and gel chromatography (data not shown) suggested that all mutant proteins retained the dimer structure. This implies that the mutations of these residues near the active sites did not cause any fundamental structural change to the B. subtilis RLP.

Changing Lys\textsuperscript{223} to alanine, aspartate, isoleucine, or glutamate resulted in complete loss of enolase activity. All mutant proteins were expressed in E. coli as soluble proteins and were purified in the same way as the wild-type recombinant protein (Fig. S1A). Native PAGE (Fig. S1B) and gel chromatography (data not shown) suggested that all mutant proteins retained the dimer structure. This implies that the mutations of these residues near the active sites did not cause any fundamental structural change to the B. subtilis RLP.

Changing Lys\textsuperscript{223} to alanine, aspartate, isoleucine, or glutamate resulted in complete loss of enolase activity. All mutant proteins were expressed in E. coli as soluble proteins and were purified in the same way as the wild-type recombinant protein (Fig. S1A). Native PAGE (Fig. S1B) and gel chromatography (data not shown) suggested that all mutant proteins retained the dimer structure. This implies that the mutations of these residues near the active sites did not cause any fundamental structural change to the B. subtilis RLP.

Changing Lys\textsuperscript{223} to alanine, aspartate, isoleucine, or glutamate resulted in complete loss of enolase activity. All mutant proteins were expressed in E. coli as soluble proteins and were purified in the same way as the wild-type recombinant protein (Fig. S1A). Native PAGE (Fig. S1B) and gel chromatography (data not shown) suggested that all mutant proteins retained the dimer structure. This implies that the mutations of these residues near the active sites did not cause any fundamental structural change to the B. subtilis RLP.

Changing Lys\textsuperscript{223} to alanine, aspartate, isoleucine, or glutamate resulted in complete loss of enolase activity. All mutant proteins were expressed in E. coli as soluble proteins and were purified in the same way as the wild-type recombinant protein (Fig. S1A). Native PAGE (Fig. S1B) and gel chromatography (data not shown) suggested that all mutant proteins retained the dimer structure. This implies that the mutations of these residues near the active sites did not cause any fundamental structural change to the B. subtilis RLP.
when Glu<sup>204</sup> was mutated to aspartate. Changing Asp<sup>203</sup> and Glu<sup>204</sup> to asparagine and glutamine, respectively, caused complete loss of activity.

The kinetic properties of D<sup>203</sup>E and E<sup>204</sup>D were compared with those of the wild-type enzyme (Fig. 3).

\[
\text{k}_{\text{cat}} \text{ and } K_m \text{ for DK-MTP-1-P of the wild-type enzyme were 153.5 s}^{-1} \text{ and } 11.7 \text{ M, respectively, under optimum conditions. Changing the carboxylmethyl group of Asp203 to a carboxyethyl group lowered } k_{\text{cat}} \text{ and } K_m \text{ for DK-MTP-1-P to } 10.8 \text{ s}^{-1} \text{ and } 9.1 \text{ M, respectively. However, shortening the side chain of Glu204 to a carboxylmethyl group caused a much smaller decrease in } k_{\text{cat}} \text{ (128.9 s}^{-1}) \text{ and a slight increase in } K_m \text{ (22.4 M). These results suggest that Asp}^{203} \text{ and Glu}^{204} \text{ are indispensable for enolase activity, but the location of the carboxyl group of Asp}^{203} \text{ has a stronger impact on catalytic efficiency than that of Glu}^{204} \text{ in the } B. \text{ subtilis RLP.}
\]

Effects of pH, CO<sub>2</sub>, and Metal Ions on B. subtilis DK-MTP-1-P Enolase Activity—Activation of RuBisCO involves CO<sub>2</sub> and Mg<sup>2+</sup> binding to the unprotonated amino group of Lys<sup>201</sup> (42). The resulting carbamatemg<sup>2+</sup> complex participates in deprotonation of C3 or enolization of RuBP. We examined the activation and/or deprotonation systems of the B. subtilis DK-MTP-1-P enolase through kinetic studies.

The pH optimum of the DK-MTP-1-P enolase reaction of the B. subtilis RLP was pH 8.2; activity at pH 7–7.2 was one-tenth that at the optimum pH (Fig. 4A). This suggests that a residue with pK<sub>a</sub> over pH 7.0 and pH 8.2 exerts a significant role in catalysis.

Since it was impossible to remove dissolved inorganic carbon in an alkaline buffer, we measured the exact amount of dissolved inorganic carbon in the reaction mixture by the reported traditional CO<sub>2</sub> removal method (39). The measured dissolved inorganic carbon after the removal was 0.21 mM, corresponding to 1.77 M CO<sub>2</sub> at the pH, ionic strength, and temperature used in these experiments (40). These values were 9.34 mM and 80.64 M when 10 mM bicarbonate was added to the N<sub>2</sub>-purged reaction mixture. The enolase activity in the low CO<sub>2</sub> buffer (1.77 M) decreased to ~77% of that of the high CO<sub>2</sub> buffer (80.64 M) (Fig. 4B).

The purified RLP enzyme was stripped of metal ions as follows. After treatment with 10 mM EDTA for 30 min at 4 °C, the enzyme was applied to a NAP-5 column (GE Healthcare) equilibrated with 50 mM Tris-HCl buffer (pH 8.2). Activity was assayed with no metals or in the presence of 1 mM various divalent metal ions. Enolase activity was undetectable after EDTA treatment, but 1 mM Mg<sup>2+</sup> recovered the original activity and saturated activity of the purified enzyme under our assay conditions (Fig. 4C). Mg<sup>2+</sup> was inhibitory at 10 mM (data not shown). Very low activities were detected with Co<sup>2+</sup>, Ni<sup>2+</sup>, and Ca<sup>2+</sup>, but Mn<sup>2+</sup>, Fe<sup>2+</sup>, Cu<sup>2+</sup>, and Zn<sup>2+</sup> were inactive (data not shown).
Similarities between Bacillus RLP and RuBisCO

Another way to analyze the functional similarity between two related enzymes is to analyze enzymatic kinetics. Even if the substrates of the two enzymes are different but similar, analyzing the effects of structural analogs of one enzyme on the catalytic activity of the other can yield information about the similarity of their substrate-binding structures.

Table 3 shows the effects of competitive inhibitors against RuBisCO on DK-MTP-1-P enolase activity of the *B. subtilis* RLP. No inhibitory effects were observed with sodium sulfate, inorganic phosphate, gluconate, and 6-phosphogluconate under these experimental conditions. However, the substrate of the RuBisCO reaction, RuBP, and its product, PGA, showed low but significant inhibitory activities. The inhibition by RuBP and PGA was competitive with respect to DK-MTP-1-P (Fig. 5, A and B). XuBP, an enantiomer of RuBP in relation to C3, is a potent inhibitor of RuBisCO but is carboxylated very slowly.

**TABLE 2**

| Mutation     | Relative activitya |
|--------------|--------------------|
| Wild type    | 100                |
| K123A        | 0.01               |
| K123N        | 0.01               |
| K123I        | 0.01               |
| K123E        | 0.01               |
| K175A        | 0.01               |
| K175I        | 0.01               |
| K175E        | 0.01               |
| K201A        | 0.01               |
| K201I        | 0.01               |
| K201E        | 0.01               |
| D203E        | 7.0                |
| D203N        | 0.01               |
| E204D        | 84.0               |
| E204Q        | 0.01               |

a The activity with wild type normalized to 100%, which corresponded to 101.3 μmol min⁻¹ mg of protein⁻¹.

**FIGURE 3.** Lineweaver-Burk plots of wild-type and mutant *B. subtilis* RLPs. Activities of wild type, D203E, and E204D of *B. subtilis* DK-MTP-1-P enolases are indicated by open circles, filled circles, and open triangles, respectively. The reaction was started by adding 1.3 μg of methylthioribulose dehydratase to a mixture containing 50 mM Tris-HCl buffer (pH 8.2), 1 mM MgCl₂, 0.9 μg DK-MTP-1-P enolase, and various amounts of MTRu-1-P. Plots are means of at least three independent measurements.

**FIGURE 4.** Effects of pH, CO₂, and metal ions on activity of *B. subtilis* DK-MTP-1-P enolase. A, enzyme activity was measured with 50 mM Tris-HCl buffer (pH 7.0–9.0). B, activity with different concentrations of CO₂. Activities were measured with CO₂-free buffer (still containing 1.8 μM CO₂; left) or 10 mM NaHCO₃ buffer (containing 80.6 μM CO₂; right). C, activity with respect to metal ions. Activities were analyzed in the presence (1 mM each) or absence (EDTA) of divalent metal ions after EDTA treatment. Other conditions were the same as in Fig. 3. Data represent mean values ± S.E. of at least three independent measurements.
**TABLE 3**

Inhibition of RLP and RuBisCO by RuBP analogs

| Inhibitor | RLP relative activity | RuBisCO $K_i$ (%) | RuBisCO $K_i$ (mM) |
|-----------|----------------------|-------------------|--------------------|
| None      | 100.0 ± 16.2         | 630 (43, 46)      |                    |
| Sodium sulfate | 98.3 ± 16.2        | 760 (43, 46)      |                    |
| Sodium dihydrogen phosphate | 107.4 ± 18.6       | 900 (43, 46)      |                    |
| Glucurate | 96.1 ± 25.9          | 1600 (43, 46)     |                    |
| 6-Phosphogluconate | 95.5 ± 13.1       | 8.5 (44, 46)      |                    |
| Xylulose 1,5-bisphosphate | 88.2 ± 13.3       | 5 (45)            |                    |
| Ribulose 1,5-bisphosphate | 84.3 ± 10.1       | 5 (45)            |                    |
| d-Phosphoglycercate | 73.7 ± 8.0         | 840 (43, 46)      |                    |
| 2-Carboxyribitol 1,5-bisphosphate | 95.9 ± 10.6       | 1.5 (30)          |                    |
| 2-Carboxyarabinitol 1,5-bisphosphate | 68.5 ± 7.7       | 0.4 (30)          |                    |

*The DK-MTP-1-P concentration was 100 mM, and the inhibitor concentration was 1 mM.*

(29). This structural analog also weakly inhibited the RLP reaction. A much stronger inhibitory effect was observed with CABP, the transition state analog of RuBisCO (Fig. 5C). The inhibition constant of CABP, 103 μm, was much lower than those of PGA and RuBP (2.29 and 10.2 mM, respectively). It should be noted that there was no significant inhibitory effect with the enantiomer of CABP with respect to C2, CRBP (Table 3). We confirmed that the activity of methylthioribulose-1-phosphate dehydratase used as the coupling enzyme in our RLP assay, was not inhibited by these inhibitors under the same conditions (data not shown).

**DISCUSSION**

There are some important similarities between RLPs and RuBisCOS, although RLPs do not fix CO$_2$ with RuBP. To date, the only metabolic reaction documented for RLPs is the DK-MTP-1-P enolization in RLPs from *B. subtilis, M. aeruginosa*, and *G. kaustophilus* in group α1 (20, 24, 25). RLPs from *R. rubrum, B. bronchiseptica, R. palustris*, and *C. tepidum* had no detectable DK-MTP-1-P enolase activity (Table 1). This is the first report analyzing the distribution of DK-MTP-1-P enolase activity among RLPs. The results clearly suggest that DK-MTP-1-P enolase activity is limited to RLPs in group α1 and that RLPs of other groups have evolved widely into enzymes catalyzing different reactions. All DK-MTP-1-P enolases have a conserved lysine at residue 123, but this residue is substituted with asparagine, glutamate, or other amino acids in RLPs with no enolase activity (Fig. 1B and Table 2). Mutations of Lys$^{123}$ to alanine, asparagine, isoleucine, or glutamate in *B. subtilis* RLP caused loss of activity (Table 2). Thus, Lys$^{123}$ is thought to be the general base to abstract the C1 proton of DK-MTP-1-P to initiate its enolization (25). However, the *R. rubrum* RuBisCO has asparagine at residue 123 but still catalyzes the DK-MTP-1-P enolase reaction, albeit at a low rate (Fig. 1B) (20). This implies that Lys$^{123}$ is not the universal general base for the DK-MTP-1-P enolase reaction in RLPs or that another residue is involved in the enolase reaction in *R. rubrum* RuBisCO.

*B. subtilis* RLP showed slightly lower activity at 1.8 μM CO$_2$ than at 80.6 μM CO$_2$ (Fig. 4B), and Mg$^{2+}$ and Mg$^{2+}$-binding carboxyl groups of Asp$^{203}$ and Glu$^{204}$ were essential for DK-MTP-1-P enolase activity (Table 2 and Fig. 4C). The present study revealed that Lys$^{201}$ is essential for the DK-MTP-1-P enolase reaction of the *B. subtilis* RLP (Table 2) and that CO$_2$ exerts a similar function in catalysis to that found in the RuBisCO reaction (Fig. 4B). Lys$^{201}$ was carbamylated in the
reported protein structure of G. kaustophilus RLP (25). The B. subtilis RLP could catalyze the enolase reaction at 1.8 μM CO₂ (Fig. 4B). Therefore, Lys²⁰¹ may be fully carbamylated at this low CO₂ concentration after binding CO₂ on its e-amino group of this amino acid residue. Considering the chemical properties of carbamate formed on an amino group, the affinity for CO₂ of this enzyme may be even higher. It may be that Lys²⁰¹ is able to localize the carboxyl groups of Asp²⁰³ and Glu²⁰⁴ and thereby position Mg²⁺ correctly in the active pocket in the absence of CO₂. The RLP mechanism of catalysis and the precise role of the carbamylated e-amino group of Lys²⁰¹ still remain to be examined.

It is generally accepted that the carbamate anion formed on the e-amino group of Lys²⁰¹ is the universal general base to abstract the C3 proton and initiate the RuBisCO reaction. The carbamylated Lys²⁰¹, therefore, can be considered as another general base in the DK-MTP-1-P enolase reaction. Accordingly, the DK-MTP-1-P enolase reaction of the B. subtilis RLP was completely inhibited by changing Lys²⁰¹ to isoleucine, glutamate, or alanine (Table 2), all of which were expected to prevent the site from forming the carboxylate structure. This observation was inconsistent with the results from the G. kaustophilus RLP, where its K201A mutant catalyzed the enolization reaction at approximately the same rate as the wild-type enzyme (25). In RuBisCOs and the G. kaustophilus RLP, the carboxylate ion formed on the Lys²⁰¹ residue also participates in coordinating the Mg²⁺ ion, in association with carboxyl groups from Asp²⁰³ and Glu²⁰⁴ (Fig. 2B). The observed inconsistency in functional significance of Lys²⁰¹ between RLPs of B. subtilis and G. kaustophilus may indicate that Lys²⁰¹ contributes to the construction of the Mg²⁺-carboxyl group complex differently in the two enzymes. There was a difference in significance of the carboxyl groups of Asp²⁰³ and Glu²⁰⁴ in B. subtilis RLP, although these carboxyl groups were essential for activity (Table 2). Since D203E showed more severe damage to its catalytic ability than E204D, the interaction between the carboxyl group of Asp²⁰³ and Mg²⁺ may be more important in catalysis. In RuBisCO, binding of the carboxyl group of Asp²⁰³ to Mg²⁺ may be firmer than that of Glu²⁰⁴ (27).

Another candidate, Lys²⁷⁵, displays an unusually low pKₐ of 7.9 in the R. rubrum RuBisCO (47). This low pKₐ agrees with the pH dependence of the B. subtilis RLP (Fig. 4A). Substituting the lysine residue with glutamate, isoleucine, or alanine removed the enolase activity completely from B. subtilis RLP (Table 2). However, because Lys²⁷⁵ is conserved in all RuBisCOs and RLPs with or without enolase activity, the exact function of this residue in the enolase reaction remains unclear (Fig. 1B and Table 1). Active site structures are highly conserved between the spinach RuBisCO and the G. kaustophilus RLP, although there is low similarity (25%) between the entire primary sequences (Figs. 1, A and B, and 28). We found that certain characteristics were shared among the active site residues of three DK-MTP-1-P enolases, the RLPs of B. subtilis, G. kaustophilus, and M. aeruginosa. The P1 (phosphate group on C1 of RuBP)-binding residues in RuBisCO, Gly²⁸¹, Gly²⁰³, and Gly²⁰⁴, were conserved, except Thr²⁴⁰ of M. aeruginosa (Fig. 1B). In contrast, the P2 (phosphate group on C5 of RuBP)-binding residues in RuBisCO, Arg²⁹⁵ and His³²⁷, were changed to hydrophobic residues in the RLPs. The catalytic residues essential for RuBP enolization in RuBisCO, Lys²¹⁷, Lys²⁰¹, Asp²⁰³, and Glu²⁰⁴ were conserved, but other catalytic residues, such as Glu⁶⁰, Asn²¹⁳, Lys²⁷⁷, and Lys³³⁸, were changed in the RLPs. This conservation of substrate-binding and catalytic residues was consistent with binding of DK-MTP-1-P and catalysis of the enolase. Intriguingly, the activity of the B. subtilis RLP was competitively inhibited by sugar bisphosphates, RuBP and CABB, with respect to the substrate DK-MTP-1-P (Table 3 and Fig. 5, A and C). This suggests that the B. subtilis RLP can bind RuBP and CABB at the active site without P2 binding residues. It is known that transition state analogs of enzymes bind to the catalytic domains with extremely high affinities (48, 49). Of all of the compounds with affinity for RuBisCO catalytic sites, the B. subtilis RLP showed strongest affinity to the transition state analog of RuBisCO, CABB (Table 3 and Fig. 5). CABB has the S-configuration about the C2 carboxyl group of CABB and was not inhibitory (Table 3 and Fig. 2A). CABB and CRBP both competitively inhibit RuBisCO, but only CABB induces a large conformational change in RuBisCO. CABB causes RuBisCO to adopt a very stable conformation after formation of the carboxylated enzyme-Mg²⁺-CABB complex (30, 50). CABB has the same steric conformation of C2 as the hydrated form of the reaction intermediate of RuBisCO, 2-carboxy-3-keto-arabinitol-1,5-bisphosphate (Fig. 2A). This explains its strong binding affinity for RuBisCO. RuBisCO discriminates CABB from CRBP via their different steric conformations. Similarly, the catalytic site of the B. subtilis RLP can bind CABB, but not CRBP, because CABB competitively inhibited the RLP reaction with its substrate in the present experiments (Table 3 and Fig. 5C). Thus, there is also an architectural similarity in catalytic sites between the B. subtilis RLP and photosynthetic RuBisCO.

REFERENCES

1. Mizioro, H. M., and Lorimer, G. H. (1983) Annu. Rev. Biochem. 52, 507–535
2. Andrews, T. J., and Lorimer, G. H. (1987) in The Biochemistry of Plants (Hatch, M. D., and Boardman, N. K., eds) Vol. 10, pp. 131–218, Academic Press, Inc., New York
3. Hartman, F. C., and Harpel, M. R. (1994) Annu. Rev. Biochem. 63, 197–234
4. Portis, A. R., Jr., and Parry, M. A. (2007) Photosynth. Res. 94, 121–143
5. Roy, H., and Andrews, T. J. (2000) in Photosynthesis: Physiology and Metabolism (Leegood, R. C., Sharkey, T. D., and von Caemmerer, S., eds) pp. 53–83, Kluwer Academic, Dordrecht, The Netherlands
6. Andrews, T. J., and Whitney, S. M. (2003) Arch. Biochem. Biophys. 414, 159–169
7. Yokota, A., and Shigeoka, S. (2008) in Advances in Plant Biochemistry and Molecular Biology (Bohnert, H., Nguyen, H., and Lewis, N., eds) Vol. 1. pp. 81–105, Elsevier Publishers, Oxford, UK
8. Feller, U., Anders, I., and Mace, T. (2008) J. Exp. Bot. 59, 1615–1624
9. Finn, M. W., and Tabita, F. R. (2003) J. Bacteriol. 185, 3049–3059
10. Selesi, D., Schmid, M., and Hartmann, A. (2005) Appl. Environ. Microbiol. 71, 175–184
11. van der Wielen, P. W. (2006) FEMS Microbiol. Lett. 259, 326–331
12. Tabita, F. R., Hanson, T. E., Li, H., Satagopan, S., Singh, J., and Chan, S. (2007) Microbiol. Mol. Biol. Rev. 71, 576–599
13. Watson, G. M., and Tabita, F. R. (1999) J. Bacteriol. 181, 1569–1575
14. Uemura, K., Anwaruzzaman Miyachi, S., and Yokota, A. (1997) Biochem. Biophys. Res. Commun. 233, 568–571
15. Tcherkez, G. G., Farquhar, G. D., and Andrews, T. J. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 7246–7251
16. Parry, M. A., Andralojc, P. J., Mitchell, R. A., Madgwick, P. J., and Keys, A. J.
