A RuBisCO-mediated carbon metabolic pathway in methanogenic archaea

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Two enzymes are considered to be unique to the photosynthetic Calvin–Benson cycle: ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), responsible for CO2 fixation, and phosphoribulokinase (PRK). Some archaea possess bona fide RuBisCOs, despite not being photosynthetic organisms, but are thought to lack PRK. Here we demonstrate the existence in methanogenic archaea of a carbon metabolic pathway involving RuBisCO and PRK, which we term ‘reductive hexulose-phosphate’ (RHP) pathway. These archaea possess both RuBisCO and a catalytically active PRK whose crystal structure resembles that of photosynthetic bacterial PRK. Capillary electrophoresis-mass spectrometric analysis of metabolites reveals that the RHP pathway, which differs from the Calvin–Benson cycle only in a few steps, is active in vivo. Our work highlights evolutionary and functional links between RuBisCO-mediated carbon metabolic pathways in methanogenic archaea and photosynthetic organisms. Whether the RHP pathway allows for autotrophy (that is, growth exclusively with CO2 as carbon source) remains unknown.

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To date, six mechanisms have been identified for carbon fixation\(^1\). Of these, members of Archaea are believed to be able to use three, namely dicarboxylate-hydroxybutyrate cycle, hydroxypropionate-hydroxybutyrate cycle, and reductive acetyl-CoA pathway. The reductive acetyl-CoA pathway has been discussed as a model of the primordial CO\(_2\)-fixing mechanism\(^1\). The Calvin–Benson cycle is the predominant photosynthetic CO\(_2\)-fixing pathway\(^2\); however, it is considered to be an evolutionary late innovation and has not been reported in Archaea\(^3\). The Calvin–Benson cycle consists of three phases: carbon fixation, carbon reduction, and ribulose-1,5-bisphosphate (RuBP) regeneration. In this process, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) fixes CO\(_2\) with RuBP to yield 3-phosphoglycerate (3-PGA), from which RuBP is regenerated\(^3\). Phosphoribulokinase (PRK) synthesizes RuBP from ribulose-5-phosphate (Ru5P) in the final step in RuBP regeneration\(^3,4\). RuBisCO and PRK are representative and unique enzymes of the photosynthetic Calvin–Benson cycle\(^6\). Interestingly, approximately half of the members of archaea that have currently been sequenced have a conserved gene for bona fide RuBisCO. Nevertheless, PRK genes and PRK activity have not been detected in Archaea. An alternative pathway distinct from the Calvin–Benson cycle may thus be present in Archaea to synthetize RuBP from either 5-phospho-D-ribose-1-pyrophosphate (PRPP) or nucleoside 5'-monophosphate (NMP) via common ribose-1,5-bisphosphate\(^6,7\). These pathways are not cyclic and cannot regenerate RuBP. The pentose bisphosphate pathway to generate RuBP as substrate for RuBisCO from nucleosides or NMP has been especially well characterized in Thermococcus kodakarensis and is thought to be involved in nucleoside assimilation and degradation\(^7\)

Here, we report a carbon metabolic pathway involving RuBisCO and PRK that can regenerate RuBP in methanogenic archaea.

Results

Enzymatic analysis of archaean PRK homologues. Some members of the archaean phylum Euryarchaeota, including Methanospirillum hungatei, possess conserved genes for PRK homologues, as well as RuBisCO. BLAST search with PRK of cyanobacterium Synechococcus PCC7942 against archaean protein database identified PRK homologues in Archaea. Photosynthetic PRKs are classified into two groups from photosynthetic bacteria and plant-type oxygenic phototrophs such as plants, algae and cyanobacteria\(^4,10,11\). Archaean PRK homologues show approximately 30% amino acid sequence identity with PRKs in photosynthetic organisms and form a clade clearly distinct from those of bacterial and plant-type PRKs in a phylogenetic tree (Fig. 1). PRK homologue genes are conserved in 11 mesophilic methanogenic archaea (Fig. 1; Supplementary Table 1). In addition to methanogenic archaea, four hyperthermophilic archaea species, including Archaeoglobus profundus placed near the base of a 16S rRNA-derived phylogenetic tree\(^12\), possess PRK homologue genes. In contrast, these genes are not conserved in the base of a 16S rRNA-derived phylogenetic tree\(^12\). We analysed the specificity of the M. hungatei PRK (MhPRK) homologue for the known P-loop kinase substrates pantothenate, thymidine, uridine, cytidine, ribosylcinnamamide, fructose 6-phosphate, AMP, ribose 5-phosphate and Ru5P (refs 14–19), but found that it showed kinase activity only for Ru5P. In an electrospray tandem mass spectrometry analysis, the product of the reaction catalysed by MhPRK with Ru5P as the substrate was identified as RuBP. This identification was based on the product’s peak (M–H)\(^-\) at m/z 309 and a comparison of its fragmentation pattern with that of authentic RuBP (Supplementary Fig. 2). MhPRK had a V\(_{\text{max}}\) of 29.28 ± 1.70 μmol min\(^{-1}\) mg protein\(^{-1}\), a K\(_{\text{m}}\) (Ru5P) of 0.28 ± 0.05 mM and a K\(_{\text{m}}\) (ATP) of 20.7 ± 1.7 μM (Table 1). PRK activity was also detected in M. hungatei cell extracts (84.14 ± 8.32 nmol min\(^{-1}\) mg protein\(^{-1}\)). Archaenal PRK homologues from Methanococcus marinigri, Methanosaeta thermophila, Methanoseta concilii, and A. profundus also showed significant PRK activities (Table 1). The V\(_{\text{max}}\) values of the archaenal PRKs were lower than those of photosynthetic PRKs, but the archaenal PRKs showed higher affinities for ATP than did the photosynthetic PRKs (Table 1). MhPRK utilized broad phosphate donor substrates, such as ATP, GTP, CTP and UTP. Kinase activities with CTP, UTP and GTP were respectively 74.3%, 46.4% and 93.96% of the ATP (Supplementary Fig. 3). On the other hand, photosynthetic PRK as a phosphate donor is relatively specific to ATP (refs 20,21).

Crystal structure of M. hungatei PRK. To structurally characterize the archaenal PRK, we determined the crystal structure of MhPRK at 2.5-Å resolution (PDB ID 5B3F). MhPRK forms a dimer with dimensions of ~105 × 50 × 40 Å\(^3\) (Supplementary Fig. 4a). The two protomers within the dimer are related by approximately two-fold noncrystallographic symmetry. Each protomer consists of an eight-stranded mixed β-sheet (β1–6 and β8–9) core surrounded by several α-helices (z1–29) and β-strands (β1', β2' and β7; Fig. 2a,c). This protomer structure is similar to that of the only photosynthetic PRK whose structure has been reported to date\(^22\), that of photosynthetic bacterial PRK from Rhodobacter sphaeroides (RsPRK; PDB ID 1A7T; r. m. s. ds of 2.1 Å over 164 topologically equivalent Cz positions of MhPRK and RsPRK) (Fig. 2a–d and Supplementary Fig. 5). This structural similarity of MhPRK and RsPRK is in spite of their low amino acid sequence identity (25%). Conversely, MhPRK (homodimer) and RsPRK (homooctamer) differ in their quaternary structures and manner of dimerization (Supplementary Fig. 4). In dimeric MhPRK, the central strands β7, which consolidates the formation of the dimer with the larger dimer interface (1694.6 Å\(^2\)), and β8 form the dimer interface. In RsPRK, strands β5, 6 and 9 and α-helix 6 participate in the formation of the dimer interface, and α-helix 7 is involved in octamer formation\(^22\). These structures involved in dimer and octamer formation are placed in the C-terminal domain. The N-terminal domain (1–198) of MhPRK resembles that of RsPRK, whereas the C-terminal domain (199–319 for MhPRK) is relatively different (Supplementary Fig. 1). Therefore, a low similarity in the C-terminal domain between MhPRK and RsPRK is consistent with differences in the manner of dimerization and quaternary structure of the two, which may lead to different enzymatic properties such as allosteric regulation of RsPRK (refs 23,24), and not MhPRK by NADH and AMP.

Two sulphate ions are positioned at the active site of the N-terminal domain at the edge of the dimer in MhPRK (Fig. 2c; Supplementary Figs 4a and 6). One interacts with Arg59, Arg62, Tyr98 and His100 side chains, and the other interacts with the main-chain amides of residues 27–31 in the P-loop along with a water molecule that interacts with the side chains of Ser31 and Thr32 (Fig. 2e; Supplementary Fig. 7a,c). Except for Arg62, these residues are highly conserved in all PRKs; they are responsible for binding of the phosphate group on Ru5P or ATP (Supplementary
Bona fide RuBisCO in M. hungatei. RufisCOs can be classified into three forms (Fig. 3): forms I and II are involved in the Calvin–Benson cycle in photosynthetic organisms\textsuperscript{30,31}, while form III is associated with the pentose bisphosphate pathway/PRPP metabolism in members of the Archaea lacking PRK (ref. 7). M. hungatei RufisCO was found to have a specific activity for the carboxylase reaction of 0.146±0.022 \text{mol min}^{-1} \text{mg protein}^{-1}. M. hungatei thus has two key enzymes in the Calvin–Benson cycle. The RufisCO of M. hungatei forms a new clade together with RufisCOs from other methanogenic archaea harbouring PRK; this clade is distinct from the form-III RufisCOs in the phylogenetic tree (Fig. 3). Archaeal form-III RufisCOs should therefore be categorized into two groups: form III-a in mainly methanogenic archaea and form III-b in other archaea. Methanogenic archaea possessing form III-a RufisCO also have PRK. The placement of
some methanogenic archaeal RuBisCOs in the form-II clade of photosynthetic bacteria reveals the evolutionary intersection between nonphotosynthetic and photosynthetic organisms with respect to RuBisCO. On the other hand, archaeal PRK forms a clade that is clearly separated from bacterial and plant-type clades (Fig. 1), which suggests that the molecular evolution of RuBisCO and PRK has been different.

A carbon metabolic pathway involving RuBisCO. The coexistence of PRK and RuBisCO indicated that CO₂ fixation might exist in *M. hungatei*. However, genes encoding three other Calvin–Benson cycle enzymes—transketolase, sedoheptulose-1,7-bisphosphatase, and ribulose-5-phosphate 3-epimerase—that catalyse the steps from fructose-6-phosphate (F6P) to Ru5P are missing from this archaeon, as they are from most members of the Archaea. In general, Archaea do not possess genes for transketolase and transaldolase needed for the non-oxidative pentose phosphate pathway, instead, aromatic amino acids are synthesized via chorismate and shikimate in the 6-deoxy-5-pentose phosphate pathway; instead, aromatic amino acids are synthesized via chorismate and shikimate in the 6-deoxy-5-pentose phosphate pathway.

To analyse the RHP pathway in vivo, a metabolomic analysis was performed with ¹³C-labelling from NaH¹³CO₃ and *M. hungatei* living cells under heterotrophic conditions. Capillary electrophoresis–mass spectrometry (CE–MS) was used to analyse the ¹³C-labelling rate of compounds. Intracellular metabolites were extracted and analysed by CE–MS after ¹³C labelling for 1, 3, 5 and 10 min to access metabolic turnover. The ratio of ¹³C to total carbon in each metabolite, the ¹³C-fraction (%), was calculated from mass isotope distribution. The ¹³C-fraction associated with sugar phosphates involved in the RHP pathway, gluconeogenesis and glycolysis increased linearly depending on time, with particularly high ¹³C-labelling rates observed for 3-PGA, fructose-1,6-bisphosphate (FBP), F6P, glucose-6-phosphate (G6P), 2-phosphoglycerate (2-PGA) and phosphoenolpyruvate (PEP) (Fig. 6; Supplementary Fig. 8). Considering that the RuBisCO was active in extracts and labelling rate and pool size of 3-PGA were high (Supplementary Fig. 9), 3-PGA was expected to be the first major compound with high ¹³C labelling rates and Ru5P showed a significant, positive correlation with ¹³C labelling rates of compounds. Intracellular metabolites were extracted and analysed by CE–MS after ¹³C labelling for 1, 3, 5 and 10 min to access metabolic turnover. The ratio of ¹³C to total carbon in each metabolite, the ¹³C-fraction (%), was calculated from mass isotope distribution. The ¹³C-fraction associated with sugar phosphates involved in the RHP pathway, gluconeogenesis and glycolysis increased linearly depending on time, with particularly high ¹³C-labelling rates observed for 3-PGA, fructose-1,6-bisphosphate (FBP), F6P, glucose-6-phosphate (G6P), 2-phosphoglycerate (2-PGA) and phosphoenolpyruvate (PEP) (Fig. 6; Supplementary Fig. 8). Considering that the RuBisCO was active in extracts and labelling rate and pool size of 3-PGA were high (Supplementary Fig. 9), 3-PGA was expected to be the first major compound with incorporation of ¹³C in *M. hungatei*. On the other hand, ¹³C atoms were incorporated into acetyl-CoA at a low rate, but hardly into pyruvate, which suggests that the reductive acetyl-CoA pathway worked slowly and that the carbon flow from acetyl-CoA to 3-PGA via pyruvate, PEP, and 2-PGA did not play a major role in ¹³C atom incorporation into 3-PGA under our experimental conditions. These results support the idea that incorporation of ¹³C in 3-PGA mainly stemmed from the carboxylase reaction of RuBisCO. Among the intermediates of the RHP pathway, FBP and F6P showed high ¹³C labelling rates and Ru5P showed a significant, albeit lower, one, strongly suggesting that this pathway was active in *M. hungatei*. The ¹³C labelling was not detected in RuBP, glyceraldehyde-3-phosphate (GAP), and dihydroxyacetone phosphate (DHAP) because the original pool sizes of these compounds are large.

### Table 1 | Enzymatic parameters of PRKs from archaea and photosynthetic organisms.

| Species | Vₘₐₓ [µmol min⁻¹ mg protein⁻¹] | Kₘ (Ru5P) [mM] | Kₘ (ATP) [µM] |
|---------|-------------------------------|----------------|---------------|
| *Methanospirillum hungatei* (Archaeon) | 29.28 ± 1.70 | 0.28 ± 0.05 | 20.70 ± 1.70 |
| *Methanoculleus marisnigri* (Archaeon) | 36.77 ± 3.09 | N.A. | N.A. |
| *Methanosaeta thermophila* (Archaeon) | 19.10 ± 0.59 | 0.23 ± 0.05 | 5.66 ± 0.21 |
| *Methanosaeta concilii* (Archaeon) | 43.84 ± 2.84 | 0.31 ± 0.08 | 17.91 ± 2.02 |
| *Archaeoglobus profundus* (Archaeon) | 1.68 ± 0.06 | N.A. | N.A. |
| *Spinacia oleracea* (Plant) | 410 ± 1 | 0.22 ± 1 | 62 ± 1 |
| *Synechococcus elongatus* PCC 7942 (Cyanobacterium) | 230 ± 1 | 0.27 ± 1 | 90 ± 1 |
| *Rhodobacter sphaeroides* (Bacterium) | 338 ± 1 | 0.10 ± 1 | 550 ± 1 |
| *Halotoleribacillus neapolitanus* (Bacterium) | 50 ± 1 | 0.24 ± 1 | 710 ± 1 |

Data for archaeal PRKs in this study are means ± s.d. of three replicates. N.A., not analysed.

* Specific activity.
† ATP concentration at half-maximal rate, as calculated from fitting ATP saturation data to the Hill equation, Runquist and Miziorka.
‡ Kobayashi et al. ATP-binding concentration at half-maximal rate, as calculated from fitting ATP saturation data to the Hill equation, MacElney et al.
unlabelled metabolites were very low (Supplementary Fig. 9). A large proportion of the $^{13}$C atoms incorporated into 3-PGA were effluxed from the RHP pathway through F6P and 3-PGA to gluconeogenesis and glycolysis, respectively. The metabolic flow of glycolysis from 3-PGA to PEP via 2-PGA was clearly detected but that from PEP to acetyl-CoA via pyruvate was not, because time course of the $^{13}$C-fraction were largely different between PEP and pyruvate. The metabolic flow of gluconeogenesis, from F6P to G6P and G1P was observed. These results suggest that a large proportion of carbons fixed by RuBisCO were supplied to gluconeogenesis and glycolysis, and a small part of these were recycled for RuBP regeneration in the RHP pathway.

Discussion

The results of our in vitro and in vivo experiments support the idea that RuBisCO and PRK function in the RHP pathway to fix CO$_2$. In addition, we found that this pathway was the major mode of CO$_2$ fixation in *M. hungatei* under our experimental heterotrophic conditions.

However, the $^{13}$C-atom labelling rate was much lower and the pool size of 3-PGA much smaller compared with observations in photosynthetic organisms$^{39,40}$. This suggests that investment of energy in the RHP pathway is much smaller compared with that invested by plants and cyanobacteria in the Calvin–Benson cycle. Methanogenic archaea carry out methanogenesis with a low energy output compared with photosystems in photosynthesis,
which is consistent with the observation that *M. hungatei* RuBisCO and PRK activities were much lower than those of photosynthetic organisms (Table 1). In the regeneration of RuBP during the RHP pathway, carbon is fixed as formaldehyde; one molecule of CO$_2$ is reduced to one molecule of formaldehyde using three molecules of ATP and two molecules of NAD(P)H (Fig. 4). Considering that methanogenic archaea thrive under extreme energy limitation, RuBP regeneration may incur a high flux of RuBP regeneration in the RHP pathway was small and insufficient for autotrophy, that is, growth exclusively with CO$_2$ as carbon source, is unknown at present.

CE–MS analysis showed that a large proportion of the carbon fixed by RuBisCO flowed to gluconeogenesis and glycolysis, and that a small fraction of this carbon was released as formaldehyde in RuBP regeneration (Fig. 6). This suggests that the metabolic flux of RuBP regeneration in the RHP pathway was small and required relatively low energy, which can be sustained by energy obtained from methanogenesis. However, the energetics of the RHP pathway in methanogenic archaea under ecological conditions remains to be addressed further.

The RHP pathway supplies fixed carbon to other important metabolic pathways. CE–MS analysis showed that the RHP pathway provides a large proportion of fixed carbon to gluconeogenesis and glycolysis via F6P and 3-PGA. The RHP pathway also provides Ru5P, which is isomerized to ribose-5-phosphate before use, for nucleotide biosynthesis (Fig. 6). Production of Ru5P is likely to be completely dependent on the RHP pathway (Fig. 4) because *M. hungatei*, like most members of the Archaea, does not contain the non-oxidative pentose phosphate pathway. These effluxes of intermediates from the RHP pathway necessitate their replenishment.

**Figure 3 | Phylogenetic tree of RuBisCOs and RuBisCO-like proteins.** The phylogenetic tree was produced using CLUSTALW. Bootstrap values were inferred from 1,000 replicates and significant bootstrapping values (> 75%) are shown on the nodes as black filled circles. RuBisCO clades are indicated as follows: green for form I, blue for form II, red for form III–a and purple for form III–b (methanogenic archaea). We propose the latter two novel small clades because form III is prominently divided. The form IV clade of RuBisCO-like proteins (RLPs), which function as enolases/isomerases in methanone recycling in some bacteria, is shown in yellow.$^6$ Species abbreviations are as follows: *C. tepidum*, *Chlorobium tepidum*; *B. subtilis*, *Bacillus subtilis*; *G. kaustophilus*, *Geobacillus kaustophilus*; *B. thuringiensis*, *Bacillus thuringiensis*; *M. burtonii*, *Methanococcus burtonii*; *M. jannaschii*, *Methanocaldococcus jannaschii*; *R. palustris*, *Rhodopseudomonas palustris* and *N. spumigena*, *Nodularia spumigena*. 

\[ 	ext{Form I} \quad \text{Form II} \quad \text{Form III–a} \quad \text{Form III–b} \quad \text{Form IV} \]

\[ \text{Plants, algae, cyanobacteria} \quad \text{Archaea} \quad \text{Photosynthetic bacteria} \]

\[ \text{R. rubrum (RLP)} \quad \text{B. thuringiensis (RLP)} \quad \text{M. palustris (RLP)} \quad \text{M. aeruginosa (RLP)} \quad \text{B. subtilis (RLP)} \]

\[ 0.1 \]

\[ \text{R. rubrum} \quad \text{B. thuringiensis} \quad \text{M. palustris} \]

\[ \text{M. acetivorans} \quad \text{M. marisnigri} \quad \text{M. tarda} \]

\[ \text{M. thermophila} \quad \text{M. harundinacea} \quad \text{M. burtonii} \quad \text{M. mahii} \quad \text{M. concilii} \quad \text{R. palustris} \]

\[ \text{M. liminatans} \quad \text{N. spumigena} \quad \text{T. kodakarensis} \quad \text{A. cryptum} \quad \text{Bradyrhizobium sp.} \]

\[ \text{A. ferrooxidans} \quad \text{P. marinus} \quad \text{A. vinosum} \quad \text{G. sulphuraria} \quad \text{M. oxyfera} \quad \text{Bradyrhizobium sp.} \]

\[ \text{A. variabilis} \quad \text{T. elongatus} \quad \text{A. thaliana} \quad \text{T. denitrificans} \quad \text{T. crunogena} \quad \text{A. veneficus} \]

\[ \text{N. hamburgensis} \quad \text{H. marismortui} \quad \text{M. jannaschii} \]

\[ \text{M. limicola} \quad \text{M. marisnigri} \quad \text{M. acetivorans} \quad \text{M. marisnigri} \quad \text{M. boonei} \quad \text{M. tarda} \]

\[ \text{M. capsulatus} \quad \text{C. tepidum} \quad \text{B. subtilis} \quad \text{B. thuringiensis} \quad \text{M. capsulatus} \]

\[ \text{Synechococcus sp.} \quad \text{P. trichocarpa} \quad \text{PCC7424} \quad \text{PCC7942} \quad \text{C. reinhardtii} \quad \text{T. elongatus} \]

\[ \text{A. veneficus} \quad \text{M. marisnigri} \quad \text{M. tarda} \quad \text{M. limicola} \quad \text{M. capsulatus} \quad \text{C. tepidum} \]

\[ \text{M. palustris} \quad \text{B. subtilis} \quad \text{B. thuringiensis} \quad \text{M. capsulatus} \]

\[ \text{Nodularia spumigena} \quad \text{Rhodopseudomonas palustris} \quad \text{N. spumigena} \]

\[ \text{Methanohalophilus mahii} \quad \text{Methanococcoides burtonii} \quad \text{M. acetivorans} \]

\[ \text{Methanosarcina acetivorans} \quad \text{Methanosarcina acetivorans} \quad \text{T. kodakarensis} \]

\[ \text{T. kodakarensis} \quad \text{M. acetivorans} \quad \text{Methanosarcina acetivorans} \quad \text{M. jannaschii} \]

\[ \text{Cyanothece sp.} \quad \text{P. marinus} \quad \text{A. ferrooxidans} \quad \text{P. marinus} \quad \text{A. vinosum} \]

\[ \text{M. acetivorans} \quad \text{M. acetivorans} \quad \text{M. marisnigri} \quad \text{M. marisnigri} \]

\[ \text{A. cryptum} \quad \text{Bradyrhizobium sp.} \quad \text{Bradyrhizobium sp.} \quad \text{Bradyrhizobium sp.} \]

\[ \text{A. profundus} \quad \text{S. oleracea} \quad \text{S. oleracea} \quad \text{S. oleracea} \]

\[ \text{A. veneficus} \quad \text{M. marisnigri} \quad \text{M. tarda} \quad \text{M. tarda} \]

\[ \text{Cyanothece sp.} \quad \text{P. marinus} \quad \text{A. ferrooxidans} \quad \text{P. marinus} \quad \text{A. vinosum} \]

\[ \text{M. acetivorans} \quad \text{M. acetivorans} \quad \text{M. marisnigri} \quad \text{M. marisnigri} \]

\[ \text{A. cryptum} \quad \text{Bradyrhizobium sp.} \quad \text{Bradyrhizobium sp.} \quad \text{Bradyrhizobium sp.} \]

\[ \text{A. veneficus} \quad \text{M. marisnigri} \quad \text{M. tarda} \quad \text{M. tarda} \]
by anaplerotic reactions. One possibility is replenishment of 3-PGA from acetyl-CoA, and *M. hungatei* possesses genes for enzymes to catalyse metabolic steps from acetyl-CoA to 3-PGA, including pyruvate ferredoxin oxidoreductase (:Mhun0450, γ subunit; :Mhun0451, δ subunit; :Mhun0452, α subunit; and :Mhun0453, β subunit), phosphoenolpyruvate synthase (:Mhun2610), phosphopyruvate hydratase (:Mhun1018, :Mhun1101 and :Mhun2893), and phosphoglycerate mutase (:Mhun0447 and :Mhun2324). However, CE–MS data suggested that this metabolic flow had little or no contribution to replenishment of 3-PGA. *M. hungatei* possesses conserved genes for the pentose bisphosphate pathway to synthesize RuBP from nucleosides or NMP for the pentose bisphosphate pathway (Supplementary Table 1), suggesting that RuBP may be provided by both the RHP pathway and the pentose bisphosphate pathway (Fig. 4). The heterotrophic medium used in our experiments provides nucleosides and NMP for the anaplerotic pentose bisphosphate pathway, to supply RuBP to the RHP pathway, as previously reported in *Thermococcus kodakar- aensis*9. Hence, the pentose bisphosphate pathway may act as an anaplerotic pathway for the RHP pathway under our experimental conditions.

Formaldehyde released from the RHP pathway can be condensed by Fae with tetrahydromethanopterin to form methylene tetrahydromethanopterin, an intermediate in both the reductive acetyl-CoA pathway and methanogenesis for energy production, another pathway for CO$_2$ fixation (Fig. 4)14,44. The *M. hungatei* HPS is fused to Fae, which might immediately capture formaldehyde35. Archaea that have genes for the RHP pathway (except *Aciduliprofundum boonei*) either possess homologous genes for HPS and Fae (for example, in *Ferroglobus placidus*, *Methanosaeta harundinacea*, *M. concilii* and *M. marisnigri*) or have a gene that encodes a fused HSP/Fae protein (in other 10 species), similar to *M. hungatei* (Supplementary Table 1). Almost all archaea harbouring the RHP pathway possess a conserved Fae/HPS fusion protein (Supplementary Table 1). The fact that HPS and Fae are fused indicates that Fae might recover formaldehyde released from the RHP pathway and supply it to the reductive acetyl-CoA pathway and methanogenesis (Fig. 4). The fusion of HPS and Fae might
minimize carbon loss and supply reduced carbon to the reductive acetyl-CoA pathway and methanogenesis. The CE–MS data showed that acetyl-CoA was not converted to 3-PGA via pyruvateferredoxin oxidoreductase, phosphoenolpyruvate synthase, phosphopyruvate hydratase, and phosphoglycerate mutase (Fig. 6). Therefore, it is expected that formaldehyde is incorporated into acetyl-CoA in the reductive acetyl-CoA pathway and then converted to lipid via the mevalonate pathway, which is the archaeal isoprenoid biosynthetic pathway. Nevertheless, the metabolomic analysis used in our study could not follow carbon from formaldehyde to methane and acetyl-CoA. To clarify these issues, a metabolomic analysis system specific for metabolites produced by these pathways will need to be established.

Many methanogenic archaea contain conserved genes for enzymes in the RHP pathway, including RuBisCO and PRK, and for the reductive acetyl-CoA pathway (Supplementary Table 1). These pathways are thus expected to be widely distributed in methanogenic archaea. Hyperthermophilic archaea of the genera Archaeoglobus and Ferroglobus also contain genes for RHP pathway enzymes (Supplementary Table 1). These archaea also have conserved genes for methanogenesis; they can produce trace

Figure 6 | Time-course analysis of the metabolite 13C fraction of M. hungatei cells. The y axis represents the ratio of 13C to total carbon in each metabolite. Data are means ± s.d. of two replicates. Ru5P, ribulose-5-phosphate; RuBP, ribulose-1,5-bisphosphate; 3-PGA, 3-phosphoglycerate; BPG, 1,3-diphosphoglycerate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; FBP, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; Hu6P, d-arabino-3-hexulose-6-phosphate; FA, formaldehyde; RSP, ribose-5-phosphate; G6P, glucose-6-phosphate; G1P, glucose-1-phosphate; 2-PGA, 2-phosphoglycerate; PEP, phosphoenolpyruvate; Pyr, pyruvate; OAA, oxaloacetate.
amounts of methane\(^6\) and have a reductive acetyl-CoA pathway\(^1\). This indicates that a metabolic link exists between the RHP pathway and the methanogenesis/reductive acetyl-CoA pathway.

Evolutionary acquisition of the photosynthetic Calvin–Benson cycle has had a major impact on the global carbon cycle\(^3\). The RHP pathway and the Calvin–Benson cycle only differ in a few steps, namely from \(\text{F6P} \) to \(\text{Ru5P}\). Thus, our results shed light on the RHP pathway and the Calvin–Benson cycle only differ in a few steps without release of carbon.

**Methods**

**Multiple sequence alignment and phylogenetic analyses.** Amino acid sequences were aligned with Genetyx ver. 11, followed by manual adjustment. Phylogenetic analyses were conducted using the neighbour-joining (NJ) method with Genetyx ver. 11. Bootstrap values were inferred from 1,000 replicates. Trees were constructed using Genetyx-tree.

**Preparation of recombinant proteins.** Expression plasmids for the His\(^-\)tagged **M. hungatei** HPS and PHI recombinant proteins were constructed as follows. DNA fragments containing genes encoding HPS (\(\text{MhHPS}, \text{MhFae-HPS}\)) and PHI (\(\text{MhPHI}\)) were amplified from genomic DNA of **M. hungatei** encoding MhFae-HPS) with the primer sets Mmprk-F and R, Mcprk-F and R, Mtprk-F and R (Supplementary Table 3), respectively. These DNA fragments with 5\'-XhoI and 3\'-BamHI sites were inserted into the pET15b vector (Novagen) using an In-Fusion HD Cloning Kit (Takara-Clontech, Japan). The PRK homo-geneous with 1 mM IPTG was induced by adding 1 mM IPTG following a temperature downshift to 15°C and cultivation at 15°C for 24 h. After His-tag purification, eluted samples were concentrated using an Amicon Ultra-10 K membrane concentrator (Merck Millipore). Buffer exchange with 50 mM Tris–HCl (pH 8.0) was carried out using a PD-10 column (GE Healthcare).

**PRK activity assay.** PRK activity was assayed at 25°C with a procedure for a cyanobacterial PRK reported previously\(^7\), with modifications. The reaction mixture contained 100 mM Tris–HCl (pH 8.0), 100 mM KCl, 0.2 mM MgCl\(_2\), 0.2 mM NADH, 2 mM ATP, 2.5 mM phosphoenolpyruvate, 2 mM RuSP, 2.5 units \(\ell\)-lactate dehydrogenase (rabbit muscle, Oriental Yeast, Japan), 1 unit pyruvate kinase (rabbit muscle, Oriental Yeast) and purified enzyme. The reaction was initiated by adding the purified enzyme, and the absorbance at 340 nm was monitored with a U3300 spectrophotometer (Hitachi, Japan).

Activity was calculated from the molecular extinction coefficient of NADH (6.22 \(\times\) 10\(^{-3}\) M \(^{-1}\) cm\(^{-1}\) at 340 nm). \(V_{\text{max}}\) and \(K_m\) values were determined from a Lineweaver–Burk plot.

In the assay for substrate specificity of **M. hungatei** PRK, RuSP was replaced with the following substrates for P-loop kinases: 6 mM phosphothreonate, 2 mM thymidine, 2 mM uridine, 2 mM riboflavinicotinamide, 2 mM fructose 6-phosphate, 2 mM AMP or 2 mM ribose 5-phosphate. Substrate concentrations were selected based on the results of previous studies\(^14\)–\(^19\).

In the assay for phosphate donor specificity of **M. hungatei** PRK, ATP was replaced with the following substrates: 2 mM CTP, 2 mM UTP or 2 mM GTP (refs 20, 21).

**Rubisco enzyme assay.** The carboxylase activity of Rubisco was measured at 25°C using a procedure modified from Pearce and Andrews\(^8\). Before the assay, **M. hungatei** Rubisco was activated by pre-incubation at 37°C for 30 min in 200 mM HEPES-KOH (pH 8.0) containing 20 mM MgCl\(_2\) and 20 mM NaHCO\(_3\). The assay mixture (500 \(\mu\)l) contained 200 mM HEPES-KOH (pH 8.0), 20 mM MgCl\(_2\), 0.2 mM NADH, 5 mM ATP, 20 mM creatine phosphate, 50 mM NaHCO\(_3\), 2 mM RuBP, 11.25 units 3-phosphoglycerate kinase (yeast, Sigma-Aldrich, Japan), 10 units glyceraldehyde 3-phosphate dehydrogenase (rabbit muscle, Sigma–Aldrich), 12.5 units creatine kinase phosphate (rabbit muscle, Oriental Yeast) and 0.05 mg carbonic anhydrase (bovine, Sigma–Aldrich). The assay mixture also included 0.05 units protocatechuate 3,4-dioxynonyanone (Pseudomonas sp.; Toyobo, Japan) and 4 mM protocatechuate to remove oxygen. All buffers were pre-swarmed with \(N_2\) and assays were carried out in septum-capped cuvettes with the headspace flushed with \(N_2\) to generate anaerobic conditions. The reaction was initiated by adding RuBP, and absorbance at 340 nm was monitored with a U3000 spectrophotometer. Activity was calculated from the molecular extinction coefficient of NADH.
Mass spectrometric analysis. The PRK reaction mixture (1 ml) comprised 30 mM phosphate buffer (pH 8.0), 10 mM MgCl₂, 2 mM ATP, 2 mM RuBP and purified MhPRK (purified at 20°C for 60 min, and then removed by terminating reactions with an Amicon Ultra-0.5 (3 K) centrifugal filter (Merck Millipore). The sample was mixed with methanol to a final concentration of 30% and injected into a Q-TOF Ultima API (Waters, MA, USA) equipped with a nanoflow probe tip (Waters). Spectra were acquired in the negative ion mode in the 50–550 Da mass range. For acquisition of tandem mass spectra, a collision energy of 15 kV was used to induce fragmentation.

Crystallization and data collection. MhPRK was expressed in BL21 (DE3) pLysS cells (Novagen), and was purified using Ni-NTA affinity chromatography and gel filtration. Selenomethionine-labelled MhPRK was expressed in B834 (DE3) cells (Novagen) grown in minimal media supplemented with amino acids and selenium-homoglutathione, and was purified using a similar protocol as for the native MhPRK. Paved, SeMet-MhPRK, and MhPRK complexes were each concentrated to approximately 10 mg ml⁻¹. The MhPRK crystals used for X-ray diffraction were grown in 4 ml solutions that contained 2 ml protein solution and 2 ml reservoir solution (20% (w/v) polyethylene glycol 4,000, 0.2 M lithium sulphate, 0.1 M HEPES sodium, pH 7.8), equilibrated against 0.5 ml reservoir solution using the hanging-drop vapour-diffusion method at 20°C. SeMet-MhPRK crystals used for X-ray diffraction were grown in 2 ml solutions containing 1 ml protein solution and 1 ml reservoir solution (1.5 M ammonium sulfate, 12% (v/v) glycerol, 0.1 M Tris–HCl, pH 8.5), equilibrated against 0.5 ml reservoir solution using the hanging-drop vapour-diffusion method at 20°C. For both proteins, the crystal that diffraction to the highest resolution was obtained by removing the crystals to 100% ethylene glycol. Crystals were each mounted into a loop, and then flash frozen in a stream of nitrogen at 100 K. Diffraction data were collected at 100 K synchrotron radiation at the SPring8 BL44XU (Japan). The diffraction data were processed using HKL2000 (ref. 53). Data collection statistics are summarized in Supplementary Table 4.

Structure determination and refinement. The MhPRK structure was solved using single-wavelength anomalous diffraction of a SeMet-MhPRK crystal. The structure was refined to 2.5-Å resolution and had R and Rfree factors of 22.2 and 28.0%, respectively (Supplementary Table 4). The crystallographic asymmetric unit contained two MhPRK protomers (chains A and B). The disordered regions for the SeMet-MhPRK datasets. Initial phases for SeMet-MhPRK were calculated and asymmetric unit were found by SHELXD (ref. 54) using the anomalous signals in the set were grown in 4 ml solutions containing 20% (v/v) polyethylene glycol 4,000, 0.2 M lithium sulphate, 0.1 M HEPES sodium, pH 7.8, equilibrated against 0.5 ml reservoir solution using the hanging-drop vapour-diffusion method at 20°C. SeMet-MhPRK crystals used for X-ray diffraction were grown in 2 ml solutions containing 1 ml protein solution and 1 ml reservoir solution (1.5 M ammonium sulfate, 12% (v/v) glycerol, 0.1 M Tris–HCl, pH 8.5), equilibrated against 0.5 ml reservoir solution using the hanging-drop vapour-diffusion method at 20°C. For both proteins, the crystal that diffraction to the highest resolution was obtained by removing the crystals to 100% ethylene glycol. Crystals were each mounted into a loop, and then flash frozen in a stream of nitrogen at 100 K. Diffraction data were collected at 100 K synchrotron radiation at the SPring8 BL44XU (Japan). The diffraction data were processed using HKL2000 (ref. 53). Data collection statistics are summarized in Supplementary Table 4.

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Author contributions
T.K. designed experiments, performed enzymatic analyses for *M. hungatei* PRK and RuBisCO together with S.M., and performed enzyme assays of PHI and HPS. T.K. and C.E. performed activity assays of other archaeal RuBisCOs. N.K., E.M. and H.M. crystallized *M. hungatei* PRK and determined its structure. M.M. and T.H. performed the metabolomic analysis with 13C labelling of *M. hungatei*. T.I. supervised the structural analysis. H.K. cultured *M. hungatei*. A.Y. and H.A. designed experiments and supervised the study. T.K., H.M. and H.A. wrote the manuscript.

Additional information
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