Substrate Induced Assembly of *Methanococoides burtonii* D-Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Dimers into Decamers.

Hernán Alonso¹, Michelle J. Blayney², Jennifer L. Beck², Spencer M. Whitney¹

¹Research School of Biology, Australian National University, PO Box 475, Canberra, Australian Capital Territory 2601, Australia. ²School of Chemistry, University of Wollongong, Wollongong, New South Wales 2522, Australia.

Running Title: Chaperone independent assembly of Rubisco decamers.

Address correspondence to: Spencer M. Whitney, Research School of Biology, Australian National University, PO Box 475, Canberra ACT 2601, Australia. Tel: +61-2-6125-5073; FAX: +61-2-6125-5075; E-mail: spencer.whitney@anu.edu.au

Like many enzymes, the biogenesis of the multi-subunit CO₂-fixing enzyme ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) in different organisms requires molecular chaperones. When expressed in *Escherichia coli*, the large (L) subunits of the Rubisco from the archaeabacterium *Methanococoides burtonii* assemble into functional dimers (L₂). However, further assembly into pentamers of L₂ (L₁₀) occurs when expressed in tobacco chloroplasts or *E. coli* producing RuBP. *In vitro* analyses indicate the sequential assembly of L₂ into L₁₀ (via detectable L₄ and L₆ intermediates) occurs without chaperone involvement and is stimulated by protein rearrangements associated with either the binding of substrate RuBP, the tight binding transition-state analog carboxyarabinitol-1,5-bisphosphate or inhibitory divalent metal ions within the active site. The catalytic properties of L₂ and L₁₀ MbR were indistinguishable. At 25°C they both shared a low specificity for CO₂ over O₂ (1.1 mol. mol⁻¹) and RuBP carboxylation rates that were distinctively enhanced at low pH (~4 s⁻¹ at pH 6, relative to 0.8 s⁻¹ at pH 8) with a temperature optimum of 55°C. Like other archaeal Rubiscos, MbR also has a high O₂ affinity (Kₘ(O₂) ~2.5 μM). The catalytic and structural similarities of MbR to other archaeal Rubiscos contrast with its closer sequence homology to bacterial L₂ Rubisco, complicating its classification within the Rubisco superfamily.

The biosynthesis and maintenance of functional enzymes is highly dependent on the assistance of molecular chaperones. Both during and after protein translation, numerous chaperone associations and disassociations prevent nascent peptides, newly synthesized peptides and even unfolded mature proteins from mis-folding into biologically non-functional products (1). The trait shared by the diverse array of molecular chaperones is their involvement in non-covalently assisting the folding/unfolding and assembly/disassembly of macromolecular structures.

The role of chaperonins in protein folding was demonstrated by showing that *E. coli* chaperonin GroEL (Cpn60) and its co-chaperonin GroES (Cpn10) promote assembly of the photosynthetic CO₂-fixing enzyme Form II ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco, EC 4.1.1.39) from the bacterium *Rhodospirillum rubrum* (2). *R. rubrum* Rubisco comprises two 50 kDa large (L) subunits that assemble head-to-tail into a dimeric (L₂) structure (3). Due to its simple structure, high level of expression in *E. coli* and innate capacity to re-assemble *in vitro*, this L₂ Rubisco remains a common substrate for examining chaperone and chaperonin function (2,4,5). This contrasts with the more common, but structurally more complex, Form I Rubiscos found in higher plants, algae, cyanobacteria, and most photo- and chemolithooauto-trophic proteobacteria. Form I Rubiscos share a hexadecameric structure comprising four L₂ catalytic dimers arranged around a 4-fold axis, and eight 13-15 kDa small (S) subunits that cap both ends of the (L₂)₄ core and influence catalysis indirectly (3). Along with their more advanced structure, the Form I enzymes have more complex assembly needs that can involve Rubisco-specific chaperones whose requirements can limit, and prevent, their assembly in both prokaryotic hosts (6) and foreign chloroplasts (7-9).

Despite their different subunit compositions and amino acid sequence, the overall...
fold of the core L₂ units in all Rubiscos are alike (3). Each L₂ contains two active (catalytic) sites located at the interface between the C-domain of one L and the N-domain of the other. Consistent with a common multi-step catalytic chemistry, the identity and position of catalytically significant residues are highly conserved (10,11). Productive binding of the 5-carbon substrate, RuBP, in the active site requires a two-step activation process: the binding of non-substrate CO₂ to a conserved lysine to form a lysyl-carbamate and its stabilisation by Mg²⁺ binding. The bound Mg²⁺ helps anchor and orientate RuBP within the active site and enables either its carboxylation (producing two 3-phosphoglycerate (3PGA) molecules) or oxygenation (producing 3PGA and 2-phosphoglycolate (2PG)). Despite their comparable active site frameworks, the CO₂-O₂ specificity (S_c/o) of Form I and II Rubiscos can vary by up to 15-fold and their RuBP carboxylation rates can differ by up to 6-fold (12,13).

In contrast to the roles of Form I and II Rubiscos in primary carbon assimilation, the Rubiscos found in some archaea appear to catalyse the removal of RuBP produced by the isomerisation of ribose-1,5-biphosphate formed during purine/pyrimidine metabolism (14,15). Like the Form II enzymes, the archaeal Rubiscos contain only L subunits assembled as either L₂ (16) or pentamers of L₂ (L₁₀) that maintain the conserved active-site framework of Rubisco (17). However, sequence comparisons group the archaeal Rubiscos as a distinctive monophyletic entity, resulting in their designation as a separate Rubisco form (Form III) (17,18). The archaeal Rubiscos characterised so far are from anoxic thermophiles and, accordingly, are highly sensitive to O₂ inhibition, display optimal activities at high temperatures and uniquely show anomalous variations in S_c/o.

Here we characterize the Rubisco enzyme from the anoxic archaea *Methanococoides burtonii* isolated from Ace Lake in Antarctica (19). Contrary to its higher sequence identity with Form II Rubisco (~40% identity to *R. rubrum*) than other archaeal Rubiscos (~35% identity to *Methanococcus jannaschii*, supplemental Fig. 1), our examination of *M. burtonii* Rubisco (MbR) produced both in *E. coli* and tobacco chloroplasts indicate that its structural and catalytic features more closely mimic other archaeal Rubiscos. We demonstrate that stable binding of sugar-phosphate ligands or inhibitory metal ions within the active site stimulates the distinctive chaperone-independent assembly of L₂ MbR into a decamer.

### Experimental procedures

**Materials—** Purified CABP (unlabelled and carboxyl-¹⁴C-labelled) and RuBP (unlabeled and ¹³H-labeled) were prepared as described (20,21). All plasmids were sequenced using BigDye terminator sequencing at the Biomolecular Resource Facility, the Australian National University. Protein content was measured using a dye-binding assay against BSA.

**Expression and purification of MbR from *E. coli*—** The *M. burtonii* Rubisco gene (*rbcL_mb*) was amplified from genomic DNA, cloned into pHUE (22) to give plasmid pHUE-MbiiL (see supplemental Fig. 2) and transformed into BL21(DE2) or BL21(DE2) transformed with the PRK-expressing plasmid pAC-BADPRK (23). The cells were grown to a OD₆₀₀ of ~0.8 in Luria-Bertani medium containing antibiotic (200 µg/mL ampicillin ± 30 µg/mL chloramphenicol) and expression of MbR and PRK induced for 16 h at 23°C with 0.5 mM IPTG and 0.25 mg/mL L-arabinose, respectively. The cells were collected by centrifugation (6000g, 6 min), the His₆-Ub-MbR fusion protein rapidly purified by IMAC and the His₆-Ub tag removed as described (22). The pure MbR was dialysed into storage buffer (20 mM HEPES-NaOH pH 7, 50 mM NaCl, 1 mM EDTA, 20% glycerol) and stored at -80°C (see supplemental Fig. 2). MbR content was quantified by CABP binding (see below). Rabbit polyclonal antisera to pure MbR was made at the Institute of Medical and Veterinary Science, Adelaide, Australia.

**MbR transformation and purification from tobacco plastids—** The tobacco plastome transforming plasmid pLevMbiiL directed the insertion of *rbcL_mb* and a promoter-less *aadA* gene (coding spectinomycin resistance) into the tobacco plastome in place of *rbcL*. pLevMbiiL was biolistically transformed into the plastome of the tobacco master line cmtrL1 as described (24) and three transplastomic tobaccoMbR transformants were grown to maturity in soil in a growth atmosphere supplemented with 2.5% (v/v) CO₂ as
described (25). The plastid-synthesized MbR was rapidly purified from young tobacco leaves by ion exchange as described (26).

**PAGE and immunoblot analysis**- Proteins were separated by SDS-PAGE (4–12% NuPAGE Bis-Tris, Invitrogen) or non-denaturing PAGE (4–12% Tris-glycine gels, Invitrogen) and visualised by Coomassie staining (Gelcode Blue, Pierce) or transferred onto nitrocellulose membrane for immunoblot analysis as described (8).

**Rubisco content, kinetics and CABP inhibition**- Maximum RuBP-dependent carboxylation rates \( (v_{\text{max}}) \) were determined as described (26). Rates of Rubisco \(^{14}\text{CO}_2\)-fixation were measured in \( \text{O}_2\)-free or air equilibrated reaction buffer (0.1 M HEPES-NaOH pH 7, 20 mM MgCl\(_2\), 10-50 mM NaH\(^{14}\text{CO}_3\)) following RuBP addition (0.5 mM) and divided by the Rubisco active site content quantified using the tight and specific binding of \(^{14}\text{C}-\text{CABP} \) (25), supplemental Fig. 3). Purified MbR was used to measure \( \text{CO}_2/\text{O}_2 \) specificity (\( S_{\text{c/o}} \)) and the Michaelis constants (\( K_m \)) for \( \text{CO}_2 \) (\( K_C \)), \( \text{O}_2 \) (\( K_O \)) and RuBP (\( K_{\text{RuBP}} \)) as described (26). The binding rate (\( k_3 \)) and inhibition constant of MbR by CABP (\( K_{\text{CABP}} \) ) and its dissociation rate (\( K_D \)) relative to purified \( \text{R. rubrum} \) (23) and tobacco (26) Rubiscos were examined in air-equilibrated buffers as described ((21); see supplemental Fig. 3). The influence of activating with Mg\(^{2+}\), Ca\(^{2+}\) or Co\(^{2+}\) on Rubisco activity, RuBP and CABP binding was examined by dialysing purified L\(_2\) MbR against nitrogen sparged buffer (20 mM HEPES-NaOH, pH 7, 50 mM NaCl, 1 mM EDTA, 20% v/v glycerol) containing Chelex resin (BioRad) for 18 hours at 4\(^\circ\)C before diluting the decarbamylated enzyme to 0.2 \( \mu \)M into reaction buffer containing 0.5 mM RuBP and 20 mM of either MgCl\(_2\), CaCl\(_2\), CoCl\(_2\) or no metal as a control.

**NanoESI-MS**—MbR was dialyzed at 4\(^\circ\)C against 10 mM ammonium acetate (NH\(_4\)OAc) and made up to 0.1 M NH\(_4\)OAc prior to analysis. The final concentrations of MbR and the other reagents (RuBP and CABP) were ~2 and 100 \( \mu \)M, respectively. Positive ion spectra were acquired using a Waters Synapt™ HDMS ESI mass spectrometer; cone voltage 200 V, trap and collision energies were both 1.0 V and backing pressure was 4.1 mBar. Spectra were acquired over the m/z range 1000-15000. The data were calibrated against a standard CsI solution (10 mg/mL) over the same m/z range.

**RESULTS**

**Methanococcoides burtonii Rubisco expressed in Escherichia coli** is \( L_2 \) - \( M. burtonii \) Rubisco (MbR) was expressed as a His\(_{6}\)-ubiquitin (H\(_6\)Ub) fusion protein in \( E. coli \) and purified to >99% purity by affinity chromatography following removal of the H\(_6\)Ub tag (supplemental Fig. 2). This purification method leaves no additional residues appended to the recombinant protein (22). Non-denaturing PAGE analysis of the purified MbR indicated it assembled as a dimer that resolved as two diffuse bands (L\(_2\), Fig. 1A). This stoichiometry was confirmed by nanoelectrospray ionisation mass spectrometry (nanoESI-MS) that revealed a subunit mass of 52,857 Da (consistent with the 52,856.5 Da predicted) and a native L\(_2\) mass of 105,714 Da (Fig. 1B).

**RuBP induces assembly of \( M. burtonii \) \( L_2 \) to \( L_{10} \)** - Incubation of L\(_2\) MbR with RuBP triggered its assembly into larger oligomers. Non-denaturing PAGE analysis of MbR incubated with RuBP showed the gradual appearance of larger molecular weight products over time and a corresponding decrease in the amount of L\(_2\) (Fig. 1C). This suggested RuBP stimulated conformational changes that triggered oligomerisation of L\(_2\) MbR into identifiable tetramers (L\(_4\)), hexamers (L\(_6\)) and decamers (L\(_{10}\)). This effect was not observed when incubated with 6-phosphogluconate, ribose-5-phosphate, fructose-6-phosphate or fructose-1,6-bisphosphate. NanoESI-MS analysis of non-activated L\(_2\) MbR incubated with RuBP confirmed its assembly into mature L\(_{10}\) complexes and showed L\(_4\) and L\(_6\) complexes that bind RuBP (Fig. 1D). This is consistent with assembly of L\(_{10}\) via intermediary L\(_4\) and L\(_6\) that are likely to be catalytically viable, but it should be noted that it is possible that some L\(_4\) and L\(_6\) may have arisen as a result of dissociation of L\(_{10}\) in the mass spectrometer.

**Stable, non-catalytic, sugar phosphate binding enhances \( L_{10} \) formation** - The assembly of L\(_2\) MbR into larger oligomers was faster using the Rubisco inhibitor 2-carboxyarabinitol-1,5-bisphosphate (CABP). Owing to its structural similarity with the transition state, CABP binds tightly to the active site of Rubiscos and prevents...
catalysis (21). For MbR, CABP binding is essentially irreversible, with a very slow CABP dissociation rate \( (K_D) \) of \( 2.0 \times 10^{-7} \text{ s}^{-1} \) that resembles that measured for tobacco Rubisco \( (K_D = 6.5 \times 10^{-7} \text{ s}^{-1}) \), supplemental Fig. 3B). Moreover, like tobacco Rubisco, MbR is strongly inhibited by CABP \( (K_d\text{CABP} = 7.8 \pm 0.1 \mu M) \), supplemental Fig. 3B) and undergoes a slow binding isomerisation step (with a rate constant of \( 0.26 \pm 0.02 \text{ s}^{-1} \), supplemental Fig. 3B) to produce the tightly-bound Rubisco-CABP complex. This tight-binding by CABP triggered conformational rearrangements that saw most \( L_2 \) MbR resolve as a defined band on native PAGE and increase its oligomeration rate (via \( L_4 \) and \( L_6 \) intermediates) into mature \( L_{10} \) complexes (Fig 1E). Consistent with this, treatment of MbR with CABP caused an association of \( L_2 \) into \( L_{10} \) MbR-CABP complexes as judged by nanoESI-MS (compare spectrum on right in Fig. 1B and spectrum in Fig 1F).

**Binding of inhibitory divalent metal ions in the active site of MbR promote \( L_{10} \) formation** - Like Form I and II Rubiscos (27,28) the binding of \( Ca^{2+} \) or \( Co^{2+} \) instead of \( Mg^{2+} \) to the conserved active site during Rubisco activation impeded \( L_2 \) MbR catalysis but not its capacity to bind RuBP. In contrast to \( Mg^{2+} \), the activation of MbR with \( Ca^{2+} \) or \( Co^{2+} \) impaired RuBP catalysis by \( \sim 90 \) and \( \sim 55\% \) (Fig. 2A), respectively, enabling the isolation of "stalled" MbR-RuBP complexes (Fig. 2B). Non-denaturing PAGE analysis of the \( Ca^{2+} \)- or \( Co^{2+} \)-bound enzymes with and without incubation with CABP showed the inhibitory metal ions alone stimulated assembly of \( L_2 \) MbR into \( L_{10} \) within 20 minutes at 25°C (Fig 2C). The formation of \( L_{10} \) complexes was not evident when \( L_2 \) MbR was first activated with \( CO_2 \) and \( Mg^{2+} \) before adding a 5-fold excess of \( Ca^{2+} \) or \( Co^{2+} \), indicating \( L_{10} \) formation was a consequence of \( Ca^{2+} \) or \( Co^{2+} \) binding to the carbamate and not elsewhere on the enzyme. The stimulated oligomerisation of \( L_2 \) MbR by \( Ca^{2+} \), and to a lesser extent \( Co^{2+} \), was also evident by nanoESI-MS (Figure 2D).

**Only \( L_{10} \) MbR is found in tobacco chloroplasts and in E. coli producing RuBP** - To investigate the influence of RuBP on MbR assembly in vivo, the enzyme was expressed in tobacco plastids. In higher plants, the L-subunit gene \( (rbcL) \) and multiple S-subunit genes \( (RbcS) \) are located in the chloroplast and nucleus, respectively. The \( rbcL \) gene was replaced with the MbR gene \( (rbcL^{MbR}) \) in the tobacco plastome by transforming it into the tobacco master line cmtrL ((24), Fig 3A). Three homoplasmic transplastomic lines (called tobacco\(^{MbR}\)) were obtained that required \( CO_2 \) supplementation to grow to reproductive maturity in soil (supplemental Fig 4). The MbR accumulated to \( \sim 8-10 \% \) (w/w) of the leaf soluble protein indicating the translation, folding and assembly requirements of this archael Rubisco are met by higher-plant chloroplasts (Fig 3B). As seen in tobacco producing \( R. rubrum \) \( L_2 \) Rubisco (25), no unassembled tobacco S-subunits were found in the tobacco\(^{MbR}\) leaves (Fig 3B). Non-denaturing PAGE showed that only \( L_{10} \) MbR was produced in the chloroplasts (Fig 3C) indicating the sustained production of RuBP by the Calvin cycle in the chloroplast stroma ensured all the \( L_{10} \) MbR was assembled into decamers.

The effect of RuBP on MbR assembly was also examined in \( E. coli \) by co-expressing \( H_6Ub-MbR \) with *Synechococcus PCC7942* phosphoribulokinase (PRK), which catalyses RuBP production from ribulose-5-phosphate. As accumulation of RuBP is toxic to \( E. coli \) (29) the co-expression of MbR was essential for cell viability. As in chloroplasts, all the \( H_6Ub-MbR \) produced in the \( E. coli \)-PRK cells was fully assembled into \( L_{10} \) complexes, confirming that RuBP stimulates the assembly of \( L_2 \) into decamers and that this assembly process is not prevented by the appended \( H_6Ub \) N-terminal sequence (Fig 3C).

**The \( L_2 \) to \( L_{10} \) transition is a reversible process** – Dialysis of purified \( L_{10} \) MbR for 24 h led to the dissociation of some decamers into \( L_2 \) (Fig 4A). At 25°C \( \sim 50\% \) of the \( L_{10} \) MbR dissociated into \( L_2 \) while only \( \sim 30\% \) of the \( L_{10} \) dissociated during dialysis at 4°C. Incubation of the dialysed enzyme with CABP (Fig 4A) stimulated re-assembly of the dissociated \( L_2 \) back into \( L_{10} \), with detectable levels of \( L_4 \) and \( L_6 \) intermediates. The assembly of \( L_2 \) into decamers therefore appears fully reversible and might occur via \( L_4 \) and \( L_6 \) transitions that may associate into \( L_{10} \) without the production of an \( L_8 \) intermediate (Fig 4B).

**The \( L_2 \) and \( L_{10} \) Rubisco have similar catalytic properties** - MbR purified form \( E. coli \) (\( L_2 \)), \( E. coli \)-PRK (\( L_{10} \)) or tobacco\(^{MbR} \) (\( L_{10} \)) was active in solutions equilibrated with ambient \( O_2 \) levels (258 \( \mu M \)) with carboxylase turnover rates \( (v_{max}) \) that ranged from \( \sim 0.5 \) to \( \sim 10 \text{ s}^{-1} \) depending on the
assay conditions. Unlike most Rubiscos, the carboxylase activity of both L2 and L10 MbR rapidly decreased at pH values above 7.0 (Fig. 5A). The enzyme showed a temperature optimum of ~55°C at pH 7 (Fig. 5B) and maximum activities at 25°C required pre-treatment of L2 and L10 MbR at 45°C for 10 min prior to assaying. Under these conditions, the \( v_{\text{c,max}} \) of L2 and L10 MbR at both 25 and 55°C were equivalent (Fig. 5B).

Comparable to the low CO\(_2\)-O\(_2\) specificities (S\(_{\text{C/O}}\)) of other archaeal Rubiscos, all three purified L2 and L10 MbR preparations had the same S\(_{\text{C/O}}\) of ~1.1 at 25°C, pH 7, that decreased to ~0.9 at 35°C (Table 1). The Michaelis constant (K\(_{\text{m}}\)) of MbR for CO\(_2\) (K\(_{\text{C}}\)) at 25°C was ~3-fold higher than the T. kodakaraensis L10 Rubisco, showing a similar affinity for CO\(_2\) to that of R. rubrum Rubisco. In agreement with the anaerobic habitat of M. burtonii, the affinity of MbR for O\(_2\) is very strong with a low K\(_{\text{m}}\) for O\(_2\) (K\(_{\text{O}}\) = 2.5±1 µM). Consequently, the measured \( v_{\text{c,max}} \) (pH 7, 25°C) under anaerobic conditions (~2 s\(^{-1}\)) was higher than that measured in ambient O\(_2\)-equilibrated buffer (~0.75 s\(^{-1}\)). Addition of a glucose oxidase/catalase O\(_2\) scavenging system to the anaerobic assays had no impact on the measured K\(_{\text{C}}\) or \( v_{\text{c,max}} \).

In accord with its proposed metabolic role in salvaging low amounts of RuBP produced during purine/pyrimidine metabolism (14,15), the K\(_{\text{m}}\) for RuBP of MbR was extremely low (0.13 ± 0.02 µM, Table 1). As this value is lower (by ~10-fold) than the inhibition constant of MbR by CABP (see above), this distinctively renders MbR more resilient to CABP inhibition in the presence of RuBP than Form I and II Rubiscos.

**DISCUSSION**

We have identified unique criteria that promote assembly of L2 MbR into decamers via a chaperone-independent process. Our *in vitro* analyses demonstrate that binding of substrate RuBP within the active site of MbR induces conformational changes in the L2 enzyme that trigger its sequential polymerisation into identifiable L4, L6 and L10 complexes (Fig 1C). The oligomerisation of L2 to L10 was faster and the yield of L10 greater when catalysis was stalled by incubating MbR with the tight binding inhibitor CABP (Fig 1E) or by activating it with the inhibitory metal ions Ca\(^{2+}\) and Co\(^{2+}\) (Fig. 2). The reversible nature of the oligomerisation process is supported by the *in vitro* partial dissociation of L10 MbR into dimers in the absence of substrate RuBP, followed by re-association of the L2 back into decamers after incubation with CABP (Fig. 4). These dramatic L2-L10 conformational changes induced by RuBP/CABP contrast with the modest transitions normally observed in other Rubiscos upon binding of these sugar phosphate ligands. These transitions include "closure" of the mobile loop 6 and the C-terminal tail over the active site, causing requisite structural rearrangements for orientating the endiol of RuBP in the active site that enable its binding of CO\(_2\) in preference to O\(_2\) at C-2 (10,11,30). Noticeably, consistent with the apparent substrate-dependent maintenance of MbR as a decamer, only L10 MbR was identified in both RuBP-producing *E. coli* and tobacco chloroplasts (Fig. 3C).

The catalytic identity between the L2 and L10 MbR complexes (Table 1) contrasts with the catalytic disparity of L8 and L8S8 Form I complexes whose carboxylase activities vary by ~100-fold (31). While the rapid association of the S-subunits to cyanobacteria Form I L8 Rubisco can occur without chaperone involvement (32), the efficient post-chaperonin assembly of the catalytically inept L8 cores shows a requirement for a Rubisco-specific chaperone RbcX (6,33). In further contrast to MbR, the assembly of L8 cores in *E. coli* occurs without the production of discernable L2, L4 or L6 intermediary complexes and occurs without the need for co-expression with RuBP (33). The catalytic uniformity of L2 and L10 MbR is also unique relative to other multi-subunit protein complexes that undergo substrate-induced oligomeric assembly (for example the chaperonin (1) and DegP chaperone (34) cages) where catalytic activity is dependent on the formation of the macromolecular structure.

The formation of L10 MbR suggests it may form a pentameric structure similar to the archaeal *T. kodakaraensis* Rubisco (17). However, a comparison of their sequences indicate that the ionic residues involved in the decamerisation of the *T. kodakaraensis* enzyme are not conserved in MbR (supplemental Fig 1), implying they are unlikely to share the same dimer-dimer interface. Indeed, MbR contains a unique insertion of 30
amino acids at the C-terminus, between the \( \beta H \) and \( \beta 7 \) secondary elements, whose influence on structure, function and oligomerisation have yet to be examined. Resolving the crystal structure of the MbR decamer is currently underway in a bid to identify the residues involved in \( L_2-L_2 \) interaction. This information (along with macromolecular structural information of \( L_2 \) MbR oligomeric assembly using single-particle cryo-electron microscopy (35)) should enable us to clarify the structure and assembly mechanism of \( L_2 \) into mature \( L_{10} \) complexes (relative to those postulated in Fig 4B) as well as provide insight as to how active-site conformational changes might promote this oligomerisation process. Understanding this process will be augmented by examining the diversity of the reversible \( L_2-L_{10} \) structural transition amongst other archaeal Rubiscos as well as elucidating if it has a biological function.

Analogous to \( R. \) rubrum Rubisco (25), the folding and assembly requirements of MbR are satisfied within tobacco chloroplasts. Although not involved in carbon assimilation in \( M. \) burtonii (see below) we uniquely show MbR is capable of supporting photosynthetic CO\(_2\)-fixation in plants. Consistent with its low \( S_{c/o} \) (~1.2) and carboxylation rate (~0.75 s\(^{-1}\)) at ambient O\(_2\), pH 7, and even less at a stromal pH of ~8, Fig 5) at 25\(^{\circ}\)C the transplastomic tobacco \#\text{Mbr} lines required CO\(_2\) supplementation (2.5 % (v/v) CO\(_2\) in air was used) for growth (supplemental Fig. 4). While the low \( S_{c/o} \) was largely the result of its low \( K_{O} \) (~2.5 \( \mu \)M), the MbR enzyme is less sensitive to \( O_2 \) inhibition than other thermophilic archaeal Rubiscos, which show little or no carboxylase activity under ambient \( O_2 \) (16,36). Importantly, the M295 and S363 residues in \( A. \) fulgidus Rubisco that influence its high \( O_2 \) affinity (36) are not conserved in MbR. Likewise, in correlation with the natural cryophilic-to-temperate habitats of \( M. \) burtonii, the optimum temperature of MbR (~55\(^{\circ}\)C) is low relative to that of thermophilic archaeal Rubiscos (~ 83 to 93\(^{\circ}\)C for \( T. \) kodakaraensis, \( M. \) jannaschii and \( A. \) fulgidus (16,18,36)). However, the characteristic high affinity for RuBP by archaeal Rubiscos is shared by MbR (Table 1) and distinctively endows MbR with a higher resilience to CABP inhibition than Form I and II Rubiscos when RuBP is present.

The high RuBP affinity of MbR is consistent with a biological RuBP-salvaging role (15). Although \( M. \) burtonii expresses MbR under normal growth conditions (37), similar to many archaea it appears to lack key Calvin-cycle enzymes such as PRK (38). However, genome analyses indicate that \( M. \) burtonii does code for an AMP phosphorylase and a ribose-1,5-bisphosphate isomerase, supporting the existence of an AMP metabolic pathway similar to that described for \( T. \) kodakaraensis, where RuBP is produced from the isomerisation of ribose-1,5-bisphosphate (15).

The \( L_{10} \) structure of MbR along with its "archaea-like" kinetic traits contrasts with its closer phylogenetic grouping with bacterial Form II Rubiscos rather than those from other archaea (supplemental Fig 1B, (18)). This conundrum raises questions with regards to the criteria used to classify different Rubisco forms. Archaeal Rubiscos were initially defined as Form III enzymes following the derivation of the novel \( T. \) kodakaraensis Rubisco pentameric ring structure and the separate grouping of archaeal Rubisco sequences (17). These defining features for the Form III enzymes are no longer absolute, and will likely become even less so as the biochemical and structural features of phylogenetically diverse archaeal Rubiscos are examined. Thus, a re-evaluation of how to best classify the different enzyme forms in the Rubisco super family might be required.

The chaperone-independent assembly and distinctive catalytic features of MbR demonstrate that there are still many things to discover about Rubisco and oligomeric enzyme assembly. The alternative selection pressures imposed on MbR during its evolution towards increased RuBP affinity rather than increased carboxylase efficiency \( (v_{c}^{\text{max}}/K_{C}) \) raises the question as to the possibility, and extent to which, appreciable improvements in \( S_{c/o} \) and \( v_{c}^{\text{max}}/K_{C} \) of MbR (and other archaeal Rubiscos) can be made by directed-evolution strategies using available biological screens (29). In conjunction with our ongoing efforts to resolve the \( L_{10} \) MbR crystal structure, such structure-function insights may prove useful towards efforts to engineer improvements into crop Rubiscos to increase productivity (12).
REFERENCES


FOOTNOTES

* We thank Prof Cavicchioli for the M. burtonii DNA and Prof George Lorimer for his comments.
This work was funded by the Australian Research Council discovery grant DP0450564 to SW.

The abbreviations used are: CABP, 2’-carboxyarabinitol-1,5-bisphosphate; HEPPS, 4-((2-hydroxyethyl)-1-piperazinepropanesulfonic acid; His6-Ub, 6x histidine tagged ubiquitin fusion peptide; IPTG, isopropyl-β-D-thiogalactopyranoside; L, Rubisco large subunit; MbR, Methanococcoides burtonii Rubisco; RuBP, D-ribulose-1,5-bisphosphate; Rubisco, RuBP carboxylase/oxygenase; S, Rubisco small subunit.

FIGURE LEGENDS

FIG. 1. Ligand induced sequential assembly of MbR L2 units at room temperature into stable decamers. Non-denaturing PAGE (panels A, C, E) and nanoESI-MS (panels B, D, F) of Rubisco complexes with the corresponding masses derived from the m/z of the ions shown in brackets. (A) Separation of purified tobacco L8S8 Rubisco (t wt), tobacco produced R. rubrum L2 Rubisco from cmtrL1 (tRr, (24)) and E. coli purified L2 MbR (EMbR, supplemental Fig. 2). (B) NanoESI-MS of the denatured EMbR monomer (5% v/v formic acid) and native dimer (0.1 M ammonium acetate, pH 7). The major ion in the spectrum of the monomer is at m/z 3524.8 corresponding to [MbR+15H]^{15+} and for the dimer it is 5564.9 corresponding to [MbR+19H]^{19+}. Induced assembly of CO2-Mg2+ activated MbR L2 units (3.8 µM final concentration) into L4, L6 and L10 complexes by (C) 1 mM RuBP and (E) 1 mM CABP over time and the corresponding nanoESI-MS of non-activated MbR after 30 min incubation with 100 µM (D) RuBP or (F) CABP. Where RuBP or CABP are present (panels D, F), the peaks in nanoESI mass spectra are broad, making it difficult to determine precise binding stoichiometries. The data were, however, consistent with the binding of one RuBP or CABP per monomer. m, marker proteins (sizes shown).

FIG. 2. The influence of activation with Mg2+, Ca2+ or Co2+ on MbR activity, sugar-phosphate binding and structure. (A) Recovery of RuBP-dependent 14CO2 activity over time relative to the activity of the Mg2+ activated MbR after 8 min. (B) Recovery of bound [14C]CABP or [3H]RuBP after activating decarbamylated L2 MbR for 15 min at 25°C with 25 mM NaHCO3 and either 20 mM MgCl2 or 20 mM CaCl2 before incubating with 2 µM of [14C]CABP (1.6 MBq/µmol, white bars) or [3H]RuBP (16 MBq µmol^{-1}, black bars) for 15 min and resolving the amount bound to MbR by Superdex G50 fine gel filtration (25). Values (± s.d.) are the average of duplicate (N = 2) samples. (C) Non-denaturing PAGE analysis of the influence of activation with Mg2+, Ca2+ or Co2+, with or without CABP, on the assembly of L2 MbR into L10 after 20 min at 25°C. m, marker proteins (sizes shown). (D) Nano ESI-MS of MbR in 100 mM ammonium acetate (pH 7) incubated for 30 min at 2°C with 100 µM of MgCl2, CaCl2 or CoCl2.

FIG. 3. RuBP stimulates assembly of L10 MbR in vivo. (A) Comparative organisation of the plastomes of wild-type tobacco (twt), the tobacco master line cmtrL1 ((24), tRr) and transplastomic tobacco producing MbR (tMbR). Plasmid pLevMbiiL was biolistically transformed into cmtrL1 where the flanking plastome sequence in pLevMbiiL (indicated by cross hatching with the numbering relative to the Nicotiana tabacum plastome sequence, genebank accession Z00044) directed the homologous replacement of the R. rubrum L2 Rubisco gene (cm rbcM), psbA 3'UTR (T) and loxP (triangle) sequences with a bicistronic gene encompassing rbcL Mb, 216-bp of the tobacco rbcL 3'UTR (T), a promoter-less aadA (marker) gene and rps16 3'UTR (t) sequence. (B) SDS PAGE and immunoblot analysis of 10 µg leaf protein from the representative tobacco lines (noting the N-terminus of MbR in tMbR, MSPQTETKASVGF, varies from the E. coli purified MbR sequence, MSLIYEDLV). (C) Non-denaturing PAGE of the samples from (B) and the purified His6-Ub-MbR expressed in RuBP producing E. coli-PRK cells before (UbL10) and after (L10) removal of the His6-Ub fusion (see supplemental Fig. 2). L8S8, Form I tobacco Rubisco; m, marker proteins (sizes shown).

FIG. 4. The L2 to L10 transition is reversible. (A) Non-denaturing PAGE analysis of purified L10 MbR (7.5 pM final concentration) before (0 h) and after 24 h of dialysis in buffer (50 mM HEPESS-NaOH pH 7.0, 20 mM EDTA) at 4°C or 22°C. After 24 h at 22°C the dialysed MbR was incubated with 20 mM...
MgCl₂ and 1 mM CABP for 30 min at 22°C prior to electrophoresis. (B) Schematic proposing a reversible assembly process of L₂ MbR via possible L₄ and L₆ intermediates into a pentameric ring.

FIG. 5. Influence of pH and temperature on MbR activity. Comparative preference of L₁₀ MbR for (A) low pH and (B) high temperature. The RuBP-carboxylase activity of L₂ (○) and L₁₀ (●) MbR at 25 and 55°C are alike. All assays were performed anaerobically.
Figure 1
Figure 2
Figure 5
TABLE I

Rubisco catalytic properties at 25°C (unless indicated otherwise)

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>Methanococcoides burtonii</th>
<th>Nicotiana tabacum</th>
<th>Rhodospirillum rubrum</th>
<th>Archaeoglobus fulgidus</th>
<th>Thermococcus kodakaraensis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L₂ (E. coli)²</td>
<td>L₁₀ (E. coli)³</td>
<td>L₁₀ (tobacco)³</td>
<td>L₂S₈ (⁴)</td>
<td>L₂ (⁴)</td>
</tr>
<tr>
<td>S&lt;sub&gt;c/o&lt;/sub&gt;</td>
<td>1.08 ± 0.04 (30°C)</td>
<td>0.88 ± 0.06 (30°C)</td>
<td>1.18 ± 0.05</td>
<td>1.14 ± 0.02</td>
<td>8.1 ± 0.2</td>
</tr>
<tr>
<td>K&lt;sub&gt;c&lt;/sub&gt; (µM)</td>
<td>n.m.</td>
<td>130 ± 4</td>
<td>n.m.</td>
<td>(11)</td>
<td>(119)</td>
</tr>
<tr>
<td>v&lt;sub&gt;c&lt;/sub&gt; max (s⁻¹)</td>
<td>1.9 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>(3.4)</td>
<td>(12)</td>
</tr>
<tr>
<td>K&lt;sub&gt;v&lt;/sub&gt; (µM)</td>
<td>n.m.</td>
<td>2.5 ± 0.3</td>
<td>n.m.</td>
<td>(259)</td>
<td>(159)</td>
</tr>
<tr>
<td>v&lt;sub&gt;v&lt;/sub&gt; max (s⁻¹)</td>
<td>n.m.</td>
<td>0.03₁</td>
<td>n.m.</td>
<td>(0.8)</td>
<td>(1.4)</td>
</tr>
<tr>
<td>K₁ RuBP (µM)</td>
<td>n.m.</td>
<td>0.13 ± 0.02</td>
<td>n.m.</td>
<td>(19)</td>
<td>(63)</td>
</tr>
</tbody>
</table>

Values shown are the average (± s.d.) of independent (N = 2 or 3) assays, n.m., not measured

¹ calculated using the equation S<sub>c/o</sub> = (v<sub>c</sub> max/Kₜ)/(v<sub>v</sub> max/K<sub>v</sub>)

² MbR purified from E. coli (supplemental Fig. 2)

³ Pure MbR from E. coli expressing PRK or tobacco MbR leaves (Fig. 3C)

⁴ In parenthesis is the published kinetic data for tobacco (N. tabacum, 8)), R. rubrum (23), A. fulgidus (36) and T. kodakaraensis (39) Rubiscos.
Substrate induced assembly of methanococoides burtonii D-Ribulose-1,5-bisphosphate carboxylase/oxygenase dimers into decamers
Hernan Alonso, Michelle J. Blayney, Jennifer L. Beck and Spencer M. Whitney

J. Biol. Chem. published online October 16, 2009

Access the most updated version of this article at doi: 10.1074/jbc.M109.050989

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2009/10/16/M109.050989.DC1