Directing the evolution of Rubisco and Rubisco activase: first impressions of a new tool for photosynthesis research

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Abstract During the last decade the practice of laboratory-directed protein evolution has become firmly established as a versatile tool in biochemical research by enabling molecular evolution toward desirable phenotypes or detection of novel structure–function interactions. Applications of this technique in the field of photosynthesis research are still in their infancy, but recently first steps have been reported in the directed evolution of the CO₂-fixing enzyme Rubisco and its helper protein Rubisco activase. Here we summarize directed protein evolution strategies and review the progressive advances that have been made to develop and apply suitable selection systems for screening mutant forms of these enzymes that improve the fitness of the host organism. The goal of increasing photosynthetic efficiency of plants by improving the kinetics of Rubisco has been a long-term goal scoring modest successes. We discuss how directed evolution methodologies may one day be able to circumvent the problems encountered during this venture.

Keywords CO₂-assimilation · Rubisco · Activase · Protein evolution · Sequence space · Mutagenesis

Abbreviations

2-PG 2-Phosphoglycolate

3-PGA	3-Phosphoglycerate
L-subunit	Large subunit
ribulose-P ₂	Ribulose-1,5-bisphosphate
Rubisco	Ribulose-P ₂ carboxylase/oxygenase
S-subunit	Small subunit

Directed evolution

The intensifying rate of scientific advancements is providing us with constantly emerging tools to explore and influence life at the molecular level. Nature's astounding diversity and ingenuity are constantly being unveiled with increasing focus placed on understanding the principles and limitations of the causative biological algorithms (Dean and Thornton 2007; Peisajovich and Tawfik 2007; Poelwijk et al. 2007). Over the last 15 years the momentum has escalated in directing the evolution of proteins toward defined purposes and has resulted in an exponential increase in publications describing the constantly developing methodologies and successful applications of this powerful research tool (see (Arnold 1998; Yuan et al. 2005; Johannes and Zhao 2006; Bershtein and Tawfik 2008) for reviews). While the common application of directed protein evolution is to generate unique and useful protein variants for a particular purpose, it is also being applied to examine the principles of protein evolution in nature (Voigt et al. 2001; Drummond et al. 2005).

The initial step in directed evolution approaches is to generate a library of mutants for which there are numerous methodologies that are generically applicable to almost every gene (Arnold and Georgiou 2003a, b; Neylon 2004). Error-prone PCR is a simple, commonly used method that introduces random copying errors during template duplication by modifying the reaction conditions (e.g., by

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adding Mn^{2+} or Mg^{2+} to the reaction) to reduce the fidelity of the DNA polymerase (Matsumura and Ellington 2002). A limitation of this method is that linked deleterious mutations make high error-rates impracticable, especially if more than one round of evolution is undertaken. Higher error rates can be achieved by DNA shuffling which involves fragmentation and random reassembly of the target gene, allowing combinations of beneficial mutations to be selected and the deleterious mutations excluded (Stemmer 1994). A variant application of DNA shuffling is family shuffling, which involves recombination of homologous gene sequences from different species which allows larger steps in sequence space to be examined (Cramer et al. 1998). If structural and/or functional information is available for the target protein to be mutated, saturation mutagenesis can be used to generate all (or most) possible mutations within a defined region (or regions) of the gene and can thus provide a faster and more effective means to evolving particular traits than DNA shuffling (Parikh and Matsumura 2005). In many applications iterative mutagenic cycles are employed to further improve the fitness of selected mutant(s).

The second step in directed evolution applications requires the development of screening applications that use either living cells or complex in vitro strategies to link changes in genotype with a measurable phenotype. The goal is to screen for fitter mutants, that is those that show improvements in a desired phenotype. This step is probably the most critical as, unlike the mutant library creation methods, the screening and selection methods are less generic with the derivation of specifically tailored methods for each protein typically required (Arnold and Georgiou 2003a, b). Moreover the screen needs to have sufficient throughput so as to be able to evaluate a suitable number of mutants. Common selection systems use in vitro methods that are capable of screening small mutant libraries ($\sim 10^2$ – 10^4 genes). These methods typically utilize multi-well plates to cultivate individually transformed host cells and assay the activity of each mutant protein separately. At the other end of the spectrum are the high-throughput in vitro screens that use compartmentalization methods which can screen for improved fitness from libraries of $>10^8$ genes (Griffiths and Tawfik 2003; Miller et al. 2006). These methods use aqueous droplets of water-in-oil emulsions to compartmentalize genes and an in vitro transcription–translation system in volumes of $\sim 10^{-15}$ l (Lin and Cornish 2002; Aharoni et al. 2005).

Where feasible the use of in vivo selection methods is favored as they provide simpler means to screen for improvements in fitness, in particular if the desired phenotype can be linked to the growth (or survival) of the selection organism. Successful application of this approach in directed evolution applications is usually limited by the

availability of, or ability to develop/engineer, a suitable selection host. However, when successfully implemented into a highly transformable organism like *E. coli*, large libraries of $\sim 10^6$ – 10^7 mutants can be screened and evaluated with ease when plated onto ~ 60 cm² of agar solidified growth media.

You get what you screened for

A fundamental outcome of directed evolution applications is that you get what you screen for (Schmidt-Dannert and Arnold 1999). Applications of directed evolution to proteins are typically aimed at modifying its catalytic performance either with respect to its native kinetic performance or toward an entirely novel enzyme function (Aharoni et al. 2005; Yoshikuni et al. 2006). Many industrial applications of directed evolution focus on adapting enzymes to function more efficiently under non-natural conditions such as increasing their thermostability or activity in unnatural solvents (Arnold et al. 2001). An agricultural application of directed evolution has made use of an evolved microbial protein that was capable of decomposing the herbicide glyphosate into four orders of magnitude more efficiently (Castle et al. 2004). Improvements in glyphosate resistance were accordingly observed in Arabidopsis, tobacco and maize lines transformed with the evolved protein.

A common (and often unanticipated) outcome of directed evolution experiments is where fitness improvements arise from mutations that, within the host organism used for selection, enhance the folding and assembly capabilities of the target protein and/or stabilize its native structure. This tendency has been widely exploited to evolve solubility and functional assembly of recalcitrant proteins that typically form insoluble, mis-folded protein aggregates in foreign organisms or in vitro transcription–translation systems (Roodveldt et al. 2005). Indeed, a wide range of in vitro and in vivo selection screens have been devised to monitor improvements in protein assembly and stability, however, the underlying *raison d'être* as to how the mutations evince these improvements is difficult to define (Waldo 2003). This contrasts with mutations that modify a protein's kinetics which can generally provide useful structure–function insights. For example, the genetic selection of chorismate mutase mutants by directed evolution has provided important novel insights into its catalytic mechanism (Woycechowsky and Hilvert 2004).

Rubisco bioselection strategies

In the research field of photosynthesis there is a rising momentum for using directed evolution methodologies to

improve the kinetics of the CO₂-fixing enzyme Ribulose 1,5-bisphosphate (ribulose-P₂) carboxylase/oxygenase (Rubisco). This highly abundant enzyme represents the only quantitatively significant interface linking the inorganic and organic phases of the biosphere's carbon cycle. The apparent kinetic shortcomings of Rubisco with regard to its poor CO₂/O₂ specificity ($S_{c/o}$) and low carboxylation efficiency have been well documented (Spreitzer and Salvucci 2002; Parry et al. 2003; Tcherkez et al. 2006) as has the modeled advantages in photosynthetic efficiency of replacing land-plant Rubiscos with kinetically superior variants (Whitney et al. 2001; Zhu et al. 2004; Long et al. 2006). Over recent decades this outlook has fueled extensive biotechnological research into improving the catalytic properties of Rubisco and to engineer such improvements into crop plants (Spreitzer and Salvucci 2002; Andrews and Whitney 2003; Parry et al. 2007).

The unveiling of Rubisco's catalytic bifunctionality revealed that in addition to catalyzing the carboxylation of ribulose-P₂ to produce two 3-phosphoglycerate (3-PGA) molecules, O₂ competed with CO₂ for fixation to ribulose-P₂ to produce 3-PGA and 2-phosphoglycolate (2-PG) (Ogren 2003). The subsequent finding that recycling of 2-PG into 3-PGA by the photorespiratory cycle led to the loss of fixed CO₂ and was energetically demanding led to initial attempts to treat Arabidopsis seed with a mutagenic agent and use a bioselection screen to identify plants with reduced ribulose-P₂ oxygenase activity (Somerville and Ogren 1982). While no Rubisco mutants were identified a large number of photorespiratory mutants were selected in addition to the ribulose-P₂ carboxylase activation deficient *rca* mutant that was later found to code for Rubisco's helper protein, Rubisco activase (Somerville et al. 1982; Salvucci et al. 1985).

The unicellular green algae *Chlamydomonas reinhardtii* has proved a useful host for Rubisco bio-selection studies particularly since its Form I structure, a hexadecamer comprising eight large (L) and eight small (S) subunits, is shared by the higher plant enzyme. The ability to maintain non-photosynthetic *C. reinhardtii* cells on media containing acetate has led to the recovery of numerous catalytically impaired Rubisco L- and S-subunit mutants (Spreitzer 1993, 1998). By using these mutants the laboratory of Robert Spreitzer has isolated numerous photosynthesis competent revertants and identified a range of compensatory mutations in both the plastome located Rubisco L-subunit gene (*rbcL*) and in the nucleus encoded S-subunit genes (*RbcS*) that enhance the catalytic properties of the Rubisco. Many mutants have been identified using this bio-selection process, which have been fundamental in highlighting catalytically important residues in Form I Rubiscos, especially those not in the direct vicinity of the L-subunit active site residues (Chen et al. 1991;

Thow et al. 1994; Du et al. 2000). Indeed these insights proved extremely valuable when integrated with phylogenetic and crystal structural comparisons as they successfully led to the rational engineering of complementary changes to the *C. reinhardtii* Rubisco L- and S-subunits that improved the $S_{c/o}$ by ~17%, albeit at the expense of reducing the ribulose-P₂ carboxylation turnover (k_c^{cat}) by ~50% (Spreitzer et al. 2005).

The natural transformation competency of many cyanobacterial species allows recombinant gene introduction using shuttle vectors and directed manipulation of their genome by homologous recombination provides strategies for metabolically engineering them toward improved Rubisco-dependency. A *Synechococcus* PCC6803 cyanobacterial mutant with its endogenous Form I enzyme substituted with the structurally simpler Form II Rubisco dimer (L₂) from the bacterium *Rhodospirillum rubrum* was made with the aim of using it to screen for spontaneous mutants with improved catalytic performance (Pierce et al. 1989). Consistent with the poorer kinetics of the *R. rubrum* enzyme the resulting cyanorubrum strain required elevated CO₂ for growth. The usefulness of this strain for identifying *R. rubrum* Rubisco mutants, or other introduced Rubiscos, with improved CO₂/O₂ specificities was not reported. A comparable bio-selection strategy for selecting kinetically varied Rubiscos using varying growth CO₂ levels can also be envisaged by coupling the mutagenic strategy used to generate a hypermutator *Synechococcus* PCC7942 strain (Emlyn-Jones et al. 2003) with a deletion mutation that renders the cyanobacterial CO₂-concentrating mechanism (CCM) inoperable (Price et al. 2008). In the hypermutator Φ (*PnirA-mutS*) PCC7942 strain the mutation rate was regulated by placing the key DNA mismatch repair *mutS* gene under the control of the *nirA* promoter whose transcription is strongly repressed when the growth nitrogen source is changed from NO₃[−] to NH₄⁺ (Emlyn-Jones et al. 2003). By coupling this inducible mutagenic strategy with a high-CO₂ requiring CCM mutation (e.g., the carboxysome deficient Δ *ccmM* strain (Ludwig et al. 2000)) it may be possible to screen for Rubisco mutants (either variants of the native enzyme or introduced foreign variants) with improvements in $S_{c/o}$ by limiting the growth CO₂ levels.

Directed evolution of Rubisco

The first report of Rubisco directed evolution applied a bio-selection strategy that used a Rubisco-deletion (Δ *rbc*) mutant of the photosynthetic bacterium *Rhodobacter capsulatus* (Paoli et al. 1998; Table 1). Trans-complementation of the mutant with libraries of XL-1Red randomly mutated *Synechococcus* PCC6301 Rubisco genes, followed by screening for photoautotrophic growth, isolated several

Table 1 Summary of key Rubisco mutants isolated by directed evolution and their biochemical phenotypes

Selection system	Rubisco examined	Number of transformants screened	Primary L-subunit mutations isolated ^a	Principal changes in biochemical phenotype	Reference
<i>Δrbc R. capsulatus</i>	<i>Synechococcus</i> PCC6301	$\sim 5 \times 10^3$	F345V (M262T, F345I)	$K_m^{\text{ribulose-P}_2}$ Reduced 50% (not examined)	(Smith and Tabita 2003)
Wild type <i>E. coli</i> - <i>Synechococcus</i> PCC7942 PRK	<i>Synechococcus</i> PCC6301	3 Generations each of $\sim 2 \times 10^5$	M262T	Carboxylation efficiency ($k_c/K_m^{\text{CO}_2}$) increased 28%; increased functional Rubisco expression in <i>E. coli</i> 5-fold	(Parikh et al. 2006; Greene et al. 2007)
MM1 ($\Delta gapA$) <i>E. coli</i> - <i>Synechococcus</i> PCC7942 PRK	<i>Rhodospirillum rubrum</i>	$\sim 5 \times 10^5$	H44N, H44Q, D117H, D117V	S_{co} reduced 40% k_c^{cat} reduced 20 to 40%	(Mueller-Cajal et al. 2007)
	<i>Synechococcus</i> PCC6301	$\sim 3 \times 10^5$	I174V, Q212L, M262T, F345L, F345I	Increased functional Rubisco expression in MM1 4 to 7-fold; $K_m^{\text{ribulose-P}_2}$ reduced 30% (F345L increased 2-fold)	(Mueller-Cajal and Whitney 2008)

^a Residue numbering is relative to the spinach Rubisco L-subunit

novel Rubisco mutants (Smith and Tabita 2003). Around 5000 transformed colonies were screened and PCC6301 Rubisco mutants that either improved or decreased cell fitness relative to those expressing the wild-type were identified depending on whether they could photoautotrophically grow in hydrogen containing 1.5 or 5% (v/v) CO₂. Only under the higher CO₂ condition could the wild-type enzyme support growth. In excess of 100 “negative” mutants (those not capable of supporting growth at 5% (v/v) CO₂) and three “positive” mutants (capable of complementing growth at 1.5% (v/v) CO₂) were identified with sequencing showing the latter mutants coded single L-subunit amino acid substitutions of either Phe-345-Val, Phe-345-Ile, or Met-262-Thr (Table 1). Detailed kinetic measurements made on the Phe-345-Val Rubisco mutant showed little change relative to wild-type except for a 50% reduction in its Michaelis constant (K_m) for ribulose-P₂ ($K_m^{\text{ribulose-P}_2}$). While this selection system provided the first step toward development of a useful screen for directed evolution of Rubisco, limitations on its throughput diminish its viability for screening large mutagenic libraries.

As indicated above, a key challenge confronting directed evolution schemes is devising an appropriate selection system where the desired phenotype can be most simply identified. The simplest schemes are therefore those that couple improved fitness with survival of the selection organism. As *Escherichia coli* is a preferred selection host in directed evolution applications because of its high transformation frequency (up to 10¹⁰ colony forming units per microgram of plasmid DNA) the challenge became how to make it dependent on Rubisco activity for growth? The concept of metabolically engineering *E. coli* toward Rubisco dependency was initially proposed by Morell et al. (1992), which involved engineering a glycolytic blockage by deleting the *E. coli* glyceraldehyde-3-phosphate dehydrogenase (*gapA*) gene and introducing a metabolic shunt comprising phosphoribulokinase (PRK) and Rubisco to by-pass the blockage (Fig. 1). PRK production in *E. coli* is, however, toxic as accumulation of its product, ribulose-P₂, is lethal to the cell and cannot be metabolized (Hudson et al. 1992). Understandably this lethality can be alleviated by co-expressing sufficient levels of functional Rubisco and this provided a relatively simple selection system to screen shuffled DNA libraries of the PCC6301 *rbcL-rbcS* operon to identify fitter Rubisco variants that supported the growth of wild-type *E. coli* producing non-permissive amounts of PRK (Parikh et al. 2006). Following three rounds of selection several Rubisco mutants which improved the fitness of the *E. coli*-PRK cells were identified, each of which shared the same Met-262-Thr L-subunit mutation isolated in the *Δrbc R. capsulatus* screen (Table 1). Biochemical characterization of the Met-262-Thr Rubisco showed that despite evincing

Fig. 1 Selection using the Rubisco-dependent *Escherichia coli* strain MM1-PRK. Blockage of the glycolytic and gluconeogenesis pathways by chromosomal deletion of the glyceraldehyde-3-phosphate dehydrogenase gene ($\Delta gapA$) produces the *E. coli* strain MM1 whose growth is dependent on being supplied with a carbon source both upstream (e.g., hexoses, glycerol) and downstream (e.g., malate, casamino acids; derived from acid hydrolyzed casein) of the deletion (dashed line) (Morell et al. 1992; Mueller-Cajar et al. 2007). This blockage can be bridged by introducing a two step metabolic shunt (diagonal hatching) comprising phosphoribulokinase (PRK, which is toxic to *E. coli* growth (Hudson et al. 1992)) and Rubisco. Using a two-plasmid expression system the expression of *Synechococcus* PCC7942 PRK from a pACYC184-based plasmid (pAC^{BAD}PRK) is controlled by the L-arabinose inducible BAD promoter (Guzman et al. 1995) and Rubisco expression is regulated by the isopropyl-beta-D-thiogalactopyranoside (IPTG) inducible *trc* promoter in pTrcHisB. Contrary to that depicted, even when producing the PRK-Rubisco shunt the MM1 cells still require limiting amounts of casamino acids and elevated CO₂ growth pressures for viability (Mueller-Cajar et al. 2007). 3-PGA, 3-phosphoglycerate; 2-PG, 2-phosphoglycolate; glc-6-P, glucose-6-phosphate; gal-3-P, glyceraldehyde-3-phosphate; gly-1,3-P₂, glyceral-1,3-bisphosphate, 3-PGA, gly-1,3-P₂, glyceral-1,3-bisphosphate; rib-5-P, D-ribulose-5-phosphate; ribulose-P₂, D-ribulose-1,5-bisphosphate; e⁻, electrons; H⁺, protons; ETP, electron transport pathway; GM, glyoxylate metabolism; OP, oxidative phosphorylation; PPP, pentose phosphate pathway; TCA, tricarboxylic acid cycle

only modest net improvements in kinetic prowess the mutation improved the fitness of the *E. coli*-PRK cells by improving L-subunit folding and assembly with the S-subunits, thus increasing Rubisco expression by ~5-fold (Greene et al. 2007).

A comparable Rubisco-dependent *E. coli* (RDE) screen using the concept of Morell et al. (1992) was developed in parallel (Mueller-Cajar et al. 2007). Expression of the *Synechococcus* PCC7942 PRK in the host $\Delta gapA$ *E. coli*

strain, MM1, is regulated by the BAD promoter to enable its regulation to be tightly modulated by the L-arabinose content in the growth media (Fig. 1). The increased susceptibility of MM1-PRK to ribulose-P₂ toxicity compared with wild-type *E. coli* augments the stringency of this RDE screen as it prevents the production of false-positives (Mueller-Cajar et al. 2007). When used to evolve *R. rubrum* Rubisco, four repeatedly isolated L-subunit point mutations (His-44-Gln/Asn or Asp-117-His/Val) were found to improve fitness in the RDE screen (Table 1). Biochemical characterization of the mutant enzymes showed that the mutations had no influence on Rubisco expression but did evince quasi-identical changes in their kinetics that included 20 to 40% reductions in k_c^{cat} and 40% decreases in CO₂/O₂ specificity. Structural analyses revealed His-44 and Asp-117 are conserved among Form II Rubiscos and form a hydrogen bond which this work showed contributes to sustaining CO₂/O₂ specificity. Based on the diminished kinetics, however, it was unclear how the mutations improved fitness to the MM1-PRK cells, as they diminished the enzymes ribulose-P₂ metabolic capacity (Mueller-Cajar et al. 2007). It was hypothesized that the increased partitioning of the substrate to 2-PG relative to 3-PGA may be metabolically favored by the cells and thus provides a possible alternative fitness solution in this RDE system other than simply increasing ribulose-P₂ turnover.

More recently the MM1-PRK RDE selection system has been used to evolve the *Synechococcus* PCC6301 enzyme under varying selection pressures by varying the growth CO₂/O₂ pressures and levels of L-arabinose in the growth media (Mueller-Cajar and Whitney 2008). In addition to the Met-262-Thr and Phe-345-Ile L-subunit mutants previously selected using the Δrbc *R. capsulatus* and *E. coli*-PRK selection systems a number of novel point mutants were predominantly selected (depending on the growth CO₂/O₂ pressures) that coded for Phe-345-Leu, Ile-174-Val, or Gln-212-Leu L-subunit substitutions (Table 1). In MM1-PRK cells these mutations improved the capacity of the L-subunit to fold and assemble correctly with the S-subunit into functional Rubisco by 4 to 7-fold. The mutations also evinced variable kinetic changes with the Gln-212-Leu substitution producing the most notable reductions in carboxylation and oxygenation efficiency and the Phe-345-Leu mutation increasing $K_m^{ribulose-P_2}$ 2-fold while in the other mutants it was reduced to ~30%. Also selected were the complementary L-subunit mutations Leu-161-Met and Met-169-Leu that together improved Rubisco yield 11-fold, while individually improving the yield by ~5-fold. It was also possible to improve functional assembly of *Synechococcus* PCC7002 Rubisco (that shows 86% identity with the PCC6301 L-subunit) in *E. coli* by 11-fold by introducing the Phe-345-Ile mutation, however, the same

substitution could not facilitate assembly of *Arabidopsis* Rubisco in the bacterium. Our understanding of Form I Rubisco assembly in *E. coli* has recently been significantly augmented by the detailed structure–function analysis of the cyanobacteria Rubisco assembly chaperone RbcX (Saschenbrecker et al. 2007). Subsequent to its folding by cpn60–cpn10 complexes, RbcX transiently interacts with the C-terminus of the Rubisco large subunit to facilitate their productive assembly prior to integration of the readily associating small subunits. Comparable studies should identify what sequences and/or chaperone processes hinder functional assembly of eukaryotic form I Rubiscos in bacterial systems (Cloney et al. 1993; Whitney et al. 2001; Whitney and Sharwood 2007). Indeed, elucidating how the mutations discovered by Mueller-Cajar and Whitney (2008) improve folding and assembly of Form I cyanobacterial Rubiscos may provide important clues toward this endeavor.

Future possibilities in directed Rubisco evolution

The unraveling of unique structure–function insights using bio-selection and directed evolution applications have established the versatility of these powerful research tools that can most likely be further exploited to unravel additional novel aspects of Rubisco's biochemistry. It is hypothesized that despite their kinetic shortcomings, all natural Rubiscos may in fact be catalytically optimized due to necessary trade-offs between catalytic turnover and CO₂/O₂ specificity (Bainbridge et al. 1995; Tcherkez et al. 2006). This has put question to the feasibility of, or the extent to which, kinetic improvements can be made to crop Rubiscos, a goal anecdotally portrayed as a holy grail quest (Morell et al. 1992; Mann 1999; Gewolb 2002). However, when one considers the single phylogenetic origin of Rubisco (Tabita et al. 2008) and the highly conserved active-site residues and architecture amongst the divergent Rubisco forms (Hanson and Tabita 2001; Andersson and Taylor 2003) the question arises as to whether more efficient Rubisco-encoding sequences exist in protein sequence space? That is, can we escape Rubisco's local optimum? It is possible that an array of multiple complementary residue changes may be needed to improve Rubiscos conserved catalytic mechanism (Andrews and Lorimer 1987; Spreitzer 1993; Watson and Tabita 1997) or even evolve a different solution. If, however, these residue changes pose unfavorable solutions in individual steps then nature's evolutionary stepwise accumulation of only beneficial or neutral mutations (Maynard Smith 1970) may have limited the variation in sequence accessible to Rubisco during its 3.5 billion year history. If so, then we should consider whether natural Rubiscos may represent partial

evolutionary solutions toward optimal kinetic efficiency. It is highly feasible that the survival dependency of photosynthetic organisms on Rubisco functionality and its high expression level may constrain the mutational tolerance of Rubisco (Drummond et al. 2005). That is, to what extent is the region of mutational diversity that current Rubiscos can sample confined by the rugged topology of its fitness landscape (Voigt et al. 2001)?

The question now becomes as to the feasibility of using high-throughput directed evolution systems (such as those outlined in Table 1) to effectively sample Rubisco's unexplored sequence space and encounter kinetically optimal variants? The enormous volume of sequence space available is clearly an impractical limitation at present and first necessitates a better understanding of Rubisco's structure–function relationships and catalytic chemistry so that the sequence space that needs to be examined can be reduced to an experimentally viable level (Voigt et al. 2001). A possible path toward improved enzymes could be the use of available Rubisco dependent selection systems (Table 1) to evolve Rubisco variants with alternative ribulose-P₂ carboxylation chemistry. Possible starting points include using a non-functional Rubisco that contains mutations at one or more conserved active site residues or Rubisco-like proteins that lack a genuine Rubisco functionality (Hanson and Tabita 2001; Ashida et al. 2003, 2005).

Directed evolution of Rubisco activase

Directed evolution proved a useful tool for improving higher plant photosynthesis under moderate heat stress by evolving more thermotolerant variants of Rubisco's accessory protein, Rubisco activase (Kurek et al. 2007). In higher plants Rubisco activase catalyses the removal of tightly bound sugar phosphates from the Rubisco active site through ATP hydrolysis to maintain Rubisco's catalytic competency (Portis 2003). Both in vitro biochemical and physiological studies showed that under moderate heat stress the functionality of Rubisco activase became impaired due to denaturation leading to Rubisco de-activation and reduced photosynthetic CO₂-assimilation rate (Crafts-Brandner and Salvucci 2000; Salvucci and Crafts-Brandner 2004; Salvucci et al. 2006). To test this hypothesis DNA shuffling was used to screen for thermostable variants of the *Arabidopsis* 42 kDa Rubisco activase isoform using multiple in vitro enzymatic methodologies (Kurek et al. 2007). Seven thermotolerant Rubisco activase variants were identified from a screen of $\sim 6.4 \times 10^3$ mutants from successive mutant libraries. Further analysis identified three lead mutants coding single (Thr-274-Arg), triple (Phe-168-Leu, Val-257-Ile, and Lys-301-Asn) or

quadruple (Phe-168-Leu, Val-257-Ile, Thr-274-Arg, and Lys-301-Asn) substitutions which were transformed into a Δrca *Arabidopsis* Rubisco activase deletion mutant. Under controlled growth conditions the mutated 42 kDa Rubisco activase enzymes significantly improved CO₂-assimilation rates, growth and seed yield in the plants exposed to periods of moderate heat stress (30°C relative to the normal growth temperature of 22°C). The ease by which the solitary Thr-274-Arg mutation to the single copy *rca* gene can increase activity and thermostability poses the question as to why nature had not selected the mutation if it is advantageous? It has been hypothesized that the heat lability of Rubisco activase is a regulatory response mechanism to inactivate Rubisco during thermal stress (Sharkey 2005). This could be to minimize Rubisco's elevated oxygenase activity at higher temperatures and thereby reduce the associated increased energy demands and carbon loss associated with the photorespiratory recycling of 2-PG. Rubisco deactivation may also serve to limit the levels of H₂O₂ produced during 2,3-pentodiulose-1,5-bisphosphate synthesis via an infrequent Rubisco oxygenase side reaction (Kim and Portis 2004). However, the proposed function of photorespiration as an energy sink during stress conditions to alleviate photoinhibitory damage to the photosynthetic apparatus (Wingler et al. 2000) implies shutting down Rubisco under moderate thermal stress would be detrimental to plant survival. Clearly growth comparisons of the transformed Δrca lines during exposure to the variable thermal stresses typical of field conditions will provide valuable insight into the viability of this strategy for improving crop yield as well as unveiling further insight into the response mechanism(s) of photosynthesis to thermal stress (Sharkey 2005).

Conclusions

Significant advances have been achieved in developing and applying screening systems to study the artificial evolution of Rubisco and Rubisco activase. Current results have highlighted numerous novel structure–function relationships, in particular identifying the functional importance of enigmatic residues found on both the L- and S-subunits. The availability of diverse mutant library creation protocols and multiple Rubisco-tailored screening and selection methods ensures that there remains an unexplored treasure trove of biochemical information to be uncovered by directed evolution, possibly even an alternative catalytic solution for driving the carboxylation of ribulose-P₂ to produce two 3-PGA molecules. The stage is now set for other areas of photosynthesis research to explore the applicability of directed evolution to modify, improve or better understand structure–function aspects of a biological

process and/or apply it for biotechnological goals. Remember, the principal hurdle is developing an appropriate selection system capable of screening sufficient number of mutants.

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References

- Aharoni A, Gaidukov L, Khersonsky O, Gould SM, Roodveldt C, Tawfik DS (2005) The 'evolvability' of promiscuous protein functions. *Nat Genet* 37:73–76
- Andersson I, Taylor TC (2003) Structural framework for catalysis and regulation in ribulose-1,5-bisphosphate carboxylase/oxygenase. *Arch Biochem Biophys* 414:130–140. doi:10.1016/S0003-9861(03)00164-4
- Andrews TJ, Lorimer GH (1987) Rubisco: structure, mechanisms, and prospects for improvement. In: Hatch MD, Boardman NK (eds) *The biochemistry of plants: a comprehensive treatise*, vol 10, photosynthesis. Academic Press, New York, pp 131–218
- Andrews TJ, Whitney SM (2003) Manipulating ribulose bisphosphate carboxylase/oxygenase in the chloroplasts of higher plants. *Arch Biochem Biophys* 414:159–169
- Arnold FH (1998) Design by directed evolution. *Acc Chem Res* 31:125–131. doi:10.1021/ar960017f
- Arnold FH, Georgiou G (2003a) Directed enzyme evolution: screening and selection methods, vol 230. Humana Press, Totowa
- Arnold FH, Georgiou G (2003b) Directed evolution library creation: methods and protocols, vol 231. Humana Press, Totowa
- Arnold FH, Wintrose PL, Miyazaki K, Gershenson A (2001) How enzymes adapt: lessons from directed evolution. *Trends Biochem Sci* 26:100–106. doi:10.1016/S0968-0004(00)01755-2
- Ashida H, Saito Y, Kojima C, Kobayashi K, Ogasawara N, Yokota A (2003) A functional link between Rubisco-like protein of *Bacillus* and photosynthetic Rubisco. *Science* 302:286–290. doi:10.1126/science.1086997
- Ashida H, Danchin A, Yokota A (2005) Was photosynthetic Rubisco recruited by acquisitive evolution from Rubisco-like proteins involved in sulfur metabolism? *Res Microbiol* 156:611–618. doi:10.1016/j.resmic.2005.01.014
- Bainbridge G, Madgwick P, Parmar S, Mitchell R, Paul M, Pitts J et al (1995) Engineering Rubisco to change its catalytic properties. *J Exp Bot* 46:1269–1276
- Bershtein S, Tawfik DS (2008) Advances in laboratory evolution of enzymes. *Curr Opin Chem Biol* 12:151–158. doi:10.1016/j.cbpa.2008.01.027
- Castle LA, Siehl DL, Gorton R, Patten PA, Chen YH, Bertain S et al (2004) Discovery and directed evolution of a glyphosate tolerance gene. *Science* 304:1151–1154. doi:10.1126/science.1096770
- Chen Z, Yu W, Lee J-H, Diao R, Spreitzer RJ (1991) Complementing amino acid substitutions within loop 6 of the α/β -barrel active site influence the CO₂/O₂ specificity of chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase. *Biochemistry* 30:8846–8850. doi:10.1021/bi00100a017
- Cloney LP, Bekkaoui DR, Hemmingsen SM (1993) Coexpression of plastid chaperonin genes and a synthetic plant Rubisco operon in *Escherichia coli*. *Plant Mol Biol* 23:1285–1290. doi:10.1007/BF00042362
- Crafts-Brandner SJ, Salvucci ME (2000) Rubisco activase constrains the photosynthetic potential of leaves at high temperature and

- CO₂. *Proc Natl Acad Sci USA* 97:13430–13435. doi: [10.1073/pnas.230451497](https://doi.org/10.1073/pnas.230451497)
- Cramer A, Raillard SA, Bermudez E, Stemmer WPC (1998) DNA shuffling of a family of genes from diverse species accelerates directed evolution. *Nature* 391:288–291. doi: [10.1038/34663](https://doi.org/10.1038/34663)
- Dean AM, Thornton JW (2007) Mechanistic approaches to the study of evolution: the functional synthesis. *Nat Rev Genet* 8:675–688. doi: [10.1038/nrg2160](https://doi.org/10.1038/nrg2160)
- Drummond DA, Bloom JD, Adami C, Wilke CO, Arnold FH (2005) Why highly expressed proteins evolve slowly. *Proc Natl Acad Sci USA* 102:14338–14343. doi: [10.1073/pnas.0504070102](https://doi.org/10.1073/pnas.0504070102)
- Du YC, Hong SJ, Spreitzer RJ (2000) RbcS suppressor mutations improve the thermal stability and CO₂/O₂ specificity of rbcL-mutant ribulose-1,5-bisphosphate carboxylase/oxygenase. *Proc Natl Acad Sci USA* 97:14206–14211. doi: [10.1073/pnas.260503997](https://doi.org/10.1073/pnas.260503997)
- Emlyn-Jones D, Price GD, Andrews TJ (2003) Nitrogen-regulated hypermutator strain of *Synechococcus* sp for use in in vivo artificial evolution. *Appl Environ Microbiol* 69:6427–6433. doi: [10.1128/AEM.69.11.6427-6433.2003](https://doi.org/10.1128/AEM.69.11.6427-6433.2003)
- Gewolb J (2002) Plant scientists see big potential in tiny plastids. *Science* 295:258–259. doi: [10.1126/science.295.5553.258](https://doi.org/10.1126/science.295.5553.258)
- Greene DN, Whitney SM, Matsumura I (2007) Artificially evolved *Synechococcus* PCC6301 Rubisco variants exhibit improvements in folding and catalytic efficiency. *Biochem J* 404:517–524. doi: [10.1042/BJ20070071](https://doi.org/10.1042/BJ20070071)
- Griffiths AD, Tawfik DS (2003) Directed evolution of an extremely fast phosphotriesterase by in vitro compartmentalization. *EMBO J* 22:24–35. doi: [10.1093/emboj/cdg014](https://doi.org/10.1093/emboj/cdg014)
- Guzman LM, Belin D, Carson MJ, Beckwith J (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J Bacteriol* 177:4121–4130
- Hanson TE, Tabita FR (2001) A ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco)-like protein from *Chlorobium tepidum* that is involved with sulfur metabolism and the response to oxidative stress. *Proc Natl Acad Sci USA* 98:4397–4402. doi: [10.1073/pnas.081610398](https://doi.org/10.1073/pnas.081610398)
- Hudson GS, Morell MK, Arvidsson YBC, Andrews TJ (1992) Synthesis of spinach phosphoribulokinase and ribulose 1,5-bisphosphate in *Escherichia coli*. *Aust J Plant Physiol* 19:213–221
- Johannes TW, Zhao HM (2006) Directed evolution of enzymes and biosynthetic pathways. *Curr Opin Microbiol* 9:261–267. doi: [10.1016/j.mib.2006.03.003](https://doi.org/10.1016/j.mib.2006.03.003)
- Kim K, Portis AR (2004) Oxygen-dependent H₂O₂ production by Rubisco. *FEBS Lett* 571:124–128. doi: [10.1016/j.febslet.2004.06.064](https://doi.org/10.1016/j.febslet.2004.06.064)
- Kurek I, Chang TK, Bertain SM, Madrigal A, Liu L, Lassner MW et al (2007) Enhanced thermostability of Arabidopsis Rubisco activase improves photosynthesis and growth rates under moderate heat stress. *Plant Cell* 19:3230–3241. doi: [10.1105/tpc.107.054171](https://doi.org/10.1105/tpc.107.054171)
- Lin HN, Cornish VW (2002) Screening and selection methods for large-scale analysis of protein function. *Angew Chem Int Ed* 41:4403–4425
- Long SP, Zhu XG, Naidu SL, Ort DR (2006) Can improvement in photosynthesis increase crop yields? *Plant Cell Environ* 29:315–330. doi: [10.1111/j.1365-3040.2005.01493.x](https://doi.org/10.1111/j.1365-3040.2005.01493.x)
- Ludwig M, Sultemeyer D, Price GD (2000) Isolation of ccmKLMN genes from the marine cyanobacterium, *Synechococcus* sp PCC7002 (Cyanobacteria), and evidence that CcmM is essential for carboxysome assembly. *J Phycol* 36:1109–1118. doi: [10.1046/j.1529-8817.2000.00028.x](https://doi.org/10.1046/j.1529-8817.2000.00028.x)
- Mann CC (1999) Genetic engineers aim to soup up crop photosynthesis. *Science* 283:314–316. doi: [10.1126/science.283.5400.314](https://doi.org/10.1126/science.283.5400.314)
- Matsumura I, Ellington AD (2002) Mutagenic PCR of protein-coding genes for in vitro evolution. In: Braman J (ed) *Methods in molecular biology*, vol 182. Humana Press, Totowa, pp 261–269
- Maynard Smith J (1970) Natural selection and concept of a protein space. *Nature* 225:563–564. doi: [10.1038/225563a0](https://doi.org/10.1038/225563a0)
- Miller OJ, Bernath K, Agresti JJ, Amitai G, Kelly BT, Mastrobattista E et al (2006) Directed evolution by in vitro compartmentalization. *Nat Methods* 3:561–570. doi: [10.1038/nmeth897](https://doi.org/10.1038/nmeth897)
- Morell MK, Paul K, Kane HJ, Andrews TJ (1992) Rubisco: maladapted or misunderstood? *Aust J Bot* 40:431–441. doi: [10.1071/BT9920431](https://doi.org/10.1071/BT9920431)
- Mueller-Cajar O, Whitney SM (2008) Evolving improved *Synechococcus* Rubisco functional expression in *Escherichia coli*. *Biochem J* (May):19. doi: [10.1042/BJ20080668](https://doi.org/10.1042/BJ20080668)
- Mueller-Cajar O, Morell M, Whitney SM (2007) Directed evolution of Rubisco in *Escherichia coli* reveals a specificity-determining hydrogen bond in the form II enzyme. *Biochemistry* 46:14067–14074. doi: [10.1021/bi700820a](https://doi.org/10.1021/bi700820a)
- Neylon C (2004) Chemical and biochemical strategies for the randomization of protein encoding DNA sequences: library construction methods for directed evolution. *Nucleic Acids Res* 32:1448–1459. doi: [10.1093/nar/gkh315](https://doi.org/10.1093/nar/gkh315)
- Ogren WL (2003) Affixing the O to Rubisco: discovering the source of photorespiratory glycolate and its regulation. *Photosynth Res* 76:53–63. doi: [10.1023/A:1024913925002](https://doi.org/10.1023/A:1024913925002)
- Paoli GC, Vichivanives P, Tabita FR (1998) Physiological control and regulation of the *Rhodobacter capsulatus* *cbb* operons. *J Bacteriol* 180:4258–4269
- Parikh MR, Matsumura I (2005) Site-saturation mutagenesis is more efficient than DNA shuffling for the directed evolution of beta-fucosidase from beta-galactosidase. *J Mol Biol* 352:621–628. doi: [10.1016/j.jmb.2005.07.020](https://doi.org/10.1016/j.jmb.2005.07.020)
- Parikh MR, Greene DN, Woods KK, Matsumura I (2006) Directed evolution of Rubisco hypermorphs through genetic selection in engineered *E. coli*. *Protein Eng Des Sel* 19:113–119. doi: [10.1093/protein/gzj010](https://doi.org/10.1093/protein/gzj010)
- Parry MAJ, Andralojc PJ, Mitchell RAC, Madgwick PJ, Keys AJ (2003) Manipulation of Rubisco: the amount, activity, function and regulation. *J Exp Bot* 54:1321–1333. doi: [10.1093/jxb/erg141](https://doi.org/10.1093/jxb/erg141)
- Parry MAJ, Madgwick PJ, Carvalho JFC, Andralojc PJ (2007) Prospects from increasing photosynthesis by overcoming the limitations of Rubisco. *J Agric Sci* 145:31–43. doi: [10.1017/S0021859606006666](https://doi.org/10.1017/S0021859606006666)
- Peisajovich SG, Tawfik DS (2007) Protein engineers turned evolutionists. *Nat Methods* 4:991–994. doi: [10.1038/nmeth1207-991](https://doi.org/10.1038/nmeth1207-991)
- Pierce J, Carlson TJ, Williams JGK (1989) A cyanobacterial mutant requiring the expression of ribulose bisphosphate carboxylase from a photosynthetic anaerobe. *Proc Natl Acad Sci USA* 86:5753–5757. doi: [10.1073/pnas.86.15.5753](https://doi.org/10.1073/pnas.86.15.5753)
- Poelwijk FJ, Kiviet DJ, Weinreich DM, Tans SJ (2007) Empirical fitness landscapes reveal accessible evolutionary paths. *Nature* 445:383–386. doi: [10.1038/nature05451](https://doi.org/10.1038/nature05451)
- Portis AR (2003) Rubisco activase—Rubisco's catalytic chaperone. *Photosynth Res* 75:11–27. doi: [10.1023/A:1022458108678](https://doi.org/10.1023/A:1022458108678)
- Price GD, Badger MR, Woodger FJ, Long BM (2008) Advances in understanding the cyanobacterial CO₂-concentrating-mechanism (CCM): functional components, C_i transporters, diversity, genetic regulation and prospects for engineering into plants. *J Exp Bot* Epub [10.1093/jxb/erm112](https://doi.org/10.1093/jxb/erm112): erm112
- Roodveldt C, Aharoni A, Tawfik DS (2005) Directed evolution of proteins for heterologous expression and stability. *Curr Opin Struct Biol* 15:50–56. doi: [10.1016/j.sbi.2005.01.001](https://doi.org/10.1016/j.sbi.2005.01.001)
- Salvucci ME, Crafts-Brandner SJ (2004) Relationship between the heat tolerance of photosynthesis and the thermal stability of Rubisco activase in plants from contrasting thermal

- environments. *Plant Physiol* 134:1460–1470. doi:[10.1104/pp.103.038323](https://doi.org/10.1104/pp.103.038323)
- Salvucci ME, Portis AR Jr, Ogren WL (1985) A soluble chloroplast protein catalyzes ribulosebiphosphate carboxylase oxygenase activation in vivo. *Photosynth Res* 7:193–201. doi:[10.1007/BF00037012](https://doi.org/10.1007/BF00037012)
- Salvucci ME, DeRidder BP, Portis AR Jr (2006) Effect of activase level and isoform on the thermotolerance of photosynthesis in *Arabidopsis* 10.1093/jxb/erl140. *J Exp Bot* 57:3793–3799
- Saschenbrecker S, Bracher A, Rao KV, Rao BV, Hartl FU, Hayer-Hartl M (2007) Structure and Function of RbcX, an assembly chaperone for hexadecameric Rubisco. *Cell* 129:1189–1200. doi:[10.1016/j.cell.2007.04.025](https://doi.org/10.1016/j.cell.2007.04.025)
- Schmidt-Dannert C, Arnold FH (1999) Directed evolution of industrial enzymes. *Trends Biotechnol* 17:135–136. doi:[10.1016/S0167-7799\(98\)01283-9](https://doi.org/10.1016/S0167-7799(98)01283-9)
- Sharkey TD (2005) Effects of moderate heat stress on photosynthesis: importance of thylakoid reactions, rubisco deactivation, reactive oxygen species, and thermotolerance provided by isoprene. *Plant Cell Environ* 28:269–277. doi:[10.1111/j.1365-3040.2005.01324.x](https://doi.org/10.1111/j.1365-3040.2005.01324.x)
- Smith SA, Tabita FR (2003) Positive and negative selection of mutant forms of prokaryotic (cyanobacterial) ribulose-1,5-bisphosphate carboxylase/oxygenase. *J Mol Biol* 331:557–569. doi:[10.1016/S0022-2836\(03\)00786-1](https://doi.org/10.1016/S0022-2836(03)00786-1)
- Somerville CR, Ogren WL (1982) Genetic modification of photorespiration. *Trends Biochem Sci* 7:171–174. doi:[10.1016/0968-0004\(82\)90130-X](https://doi.org/10.1016/0968-0004(82)90130-X)
- Somerville CR, Portis AR Jr, Ogren WL (1982) A mutant of *Arabidopsis thaliana* which lacks activation of RuBP carboxylase in vivo. *Plant Physiol* 70:381–387
- Spreitzer RJ (1993) Genetic dissection of Rubisco structure and function. *Annu Rev Plant Physiol Plant Mol Biol* 44:411–434. doi:[10.1146/annurev.pp.44.060193.002211](https://doi.org/10.1146/annurev.pp.44.060193.002211)
- Spreitzer RJ (1998) Genetic engineering of Rubisco. In: Rochaix JD, Goldschmidt-Clermont M, Merchant S (eds) *The molecular biology of chloroplasts and mitochondria in Chlamydomonas*. Kluwer Academic Publishers, Dordrecht, pp 515–527
- Spreitzer RJ, Salvucci ME (2002) Rubisco: structure, regulatory interactions, and possibilities for a better enzyme. *Annu Rev Plant Biol* 53:449–475. doi:[10.1146/annurev.arplant.53.100301.135233](https://doi.org/10.1146/annurev.arplant.53.100301.135233)
- Spreitzer RJ, Peddi SR, Satagopan S (2005) Phylogenetic engineering at an interface between large and small subunits imparts land-plant kinetic properties to algal Rubisco. *Proc Natl Acad Sci USA* 102:17225–17230. doi:[10.1073/pnas.0508042102](https://doi.org/10.1073/pnas.0508042102)
- Stemmer WPC (1994) DNA shuffling by random fragmentation and reassembly—in-vitro recombination for molecular evolution. *Proc Natl Acad Sci USA* 91:10747–10751. doi:[10.1073/pnas.91.22.10747](https://doi.org/10.1073/pnas.91.22.10747)
- Tabita FR, Satagopan S, Hanson TE, Kreel NE, Scott SS (2008) Distinct form I, II, III, and IV Rubisco proteins from the three kingdoms of life provide clues about Rubisco evolution and structure/function relationships. *J Exp Bot* 59(7):1515–1524. erm361
- Tcherkez GB, Farquhar GD, Andrews TJ (2006) Despite slow catalysis and confused substrate specificity, all ribulose biphosphate carboxylases may be nearly perfectly optimized. *Proc Natl Acad Sci USA* 103:7246–7251. doi:[10.1073/pnas.0600605103](https://doi.org/10.1073/pnas.0600605103)
- Thow G, Zhu G, Spreitzer RJ (1994) Complementing substitutions within loop regions 2 and 3 of the α/β -barrel active site influence the CO₂/O₂ specificity of chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase. *Biochemistry* 33:5109–5114. doi:[10.1021/bi00183a014](https://doi.org/10.1021/bi00183a014)
- Voigt CA, Kauffman S, Wang ZG (2001) Rational evolutionary design: the theory of in vitro protein evolution. *Adv Protein Chem* 55:79–160
- Waldo GS (2003) Genetic screens and directed evolution for protein solubility. *Curr Opin Chem Biol* 7:33–38. doi:[10.1016/S1367-5931\(02\)00017-0](https://doi.org/10.1016/S1367-5931(02)00017-0)
- Watson GMF, Tabita FR (1997) Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: a molecule for phylogenetic and enzymological investigation. *FEMS Microbiol Lett* 146:13–22. doi:[10.1111/j.1574-6968.1997.tb10165.x](https://doi.org/10.1111/j.1574-6968.1997.tb10165.x)
- Whitney SM, Sharwood RE (2007) Linked Rubisco subunits can assemble into functional oligomers without impeding catalytic performance. *J Biol Chem* 282:3809–3818. doi:[10.1074/jbc.M610479200](https://doi.org/10.1074/jbc.M610479200)
- Whitney SM, Baldet P, Hudson GS, Andrews TJ (2001) Form I Rubiscos from non-green algae are expressed abundantly but not assembled in tobacco chloroplasts. *Plant J* 26:535–547. doi:[10.1046/j.1365-313x.2001.01056.x](https://doi.org/10.1046/j.1365-313x.2001.01056.x)
- Wingler A, Lea PJ, Quick WP, Leegood RC (2000) Photorespiration: metabolic pathways and their role in stress protection. *Philos Trans R Soc Lond B Biol Sci* 355:1517–1529. doi:[10.1098/rstb.2000.0712](https://doi.org/10.1098/rstb.2000.0712)
- Woycechowsky KJ, Hilvert D (2004) Deciphering enzymes—genetic selection as a probe of structure and mechanism. *Eur J Biochem* 271:1630–1637. doi:[10.1111/j.1432-1033.2004.04073.x](https://doi.org/10.1111/j.1432-1033.2004.04073.x)
- Yoshikuni Y, Ferrin TE, Keasling JD (2006) Designed divergent evolution of enzyme function. *Nature* 440:1078–1082. doi:[10.1038/nature04607](https://doi.org/10.1038/nature04607)
- Yuan L, Kurek I, English J, Keenan R (2005) Laboratory-directed protein evolution. *Microbiol Mol Biol Rev* 69:373. doi:[10.1128/MMBR.69.3.373-392.2005](https://doi.org/10.1128/MMBR.69.3.373-392.2005)
- Zhu XG, Portis AR, Long SP (2004) Would transformation of C₃ crop plants with foreign Rubisco increase productivity? A computational analysis extrapolating from kinetic properties to canopy photosynthesis. *Plant Cell Environ* 27:155–165. doi:[10.1046/j.1365-3040.2004.01142.x](https://doi.org/10.1046/j.1365-3040.2004.01142.x)