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

3-PGA 3-Phosphoglycerate
L-subunit Large subunit
ribulose-P₂ Ribulose-1,5-bisphosphate
Rubisco Ribulose-P₂ 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
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 (\(\sim 10^2–10^5\) 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 \(\sim 60\,\text{cm}^2\) 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 (Wald 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 CO$_2$-fixing enzyme Ribulose 1,5-bisphosphate (ribulose-P$_2$) carboxylase/oxygenase (Rubisco). This highly abundant enzyme represents the only quantitatively significant interface linking the inorganic and organic phases of the biospheres carbon cycle. The apparent kinetic shortcomings of Rubisco with regard to its poor CO$_2$/O$_2$ specificity ($S_{i/o}$) 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-P$_2$ to produce two 3-phosphoglycerate (3-PGA) molecules, O$_2$ competed with CO$_2$ for fixation to ribulose-P$_2$ 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 CO$_2$ 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-P$_2$ 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-P$_2$ carboxylase activation deficient rca mutant that was later found to code for Rubisco’s helper protein, Rubisco activase (Somerville and Salvucci 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 $S_{i/o}$ by $\sim 17\%$, albeit at the expense of reducing the ribulose-P$_2$ carboxylation turnover ($k_c^{cat}$) by $\sim 50\%$ (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 (L$_2$) 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 cyano-rubrum strain required elevated CO$_2$ for growth. The usefullness of this strain for identifying R. rubrum Rubisco mutants, or other introduced Rubiscos, with improved CO$_2$/O$_2$ specificities was not reported. A comparable bio-selection strategy for selecting kinetically varied Rubiscos using varying growth CO$_2$ 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 CO$_2$-concentrating mechanism (CCM) inoperable (Price et al. 2008). In the hypermutator $\Phi$(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 NO$_3^-$ to NH$_4^+$ (Emlyn-Jones et al. 2003). By coupling this inducible mutagenic strategy with a high-CO$_2$ requiring CCM mutation (e.g., the carboxysome deficient $\Delta$cmm 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 $S_{i/o}$ by limiting the growth CO$_2$ levels.

**Directed evolution of Rubisco**

The first report of Rubisco directed evolution applied a bio-selection strategy that used a Rubisco-deletion ($\Delta$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
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 ($K_m$ ribulose-P$_2$). While this selection system provided the first step toward development of an 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 $10^{10}$ 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 ($\text{gapA}$) 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-P$_2$, 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 $\text{rbcL-rbcS}$ 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 $\Delta rbc R. capsulatus$ screen (Table 1). Biochemical characterization of the Met-262-Thr Rubisco showed that despite evincing
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 ΔgapA 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 $k_{\text{cat}}^\text{max}$ 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 $K_m^\text{Ribulose-P₂}$ 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 preserved 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 thermostolerant 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 thermostolerant Rubisco activase variants were identified from a screen of ~6.4 x 10⁴ 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 \( \Delta rca \) Arabidopsis Rubisco activase deletion mutant. Under controlled growth conditions the mutated 42 kDa Rubisco activase enzymes significantly improved CO\(_2\)-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 photosynthetic recycling of 2-PG. Rubisco deactivation may also serve to limit the levels of \( \text{H}_2\text{O}_2 \) 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 \( \Delta rca \) 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-P\(_2\) 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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