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1 Short title: Enzyme complexes for metabolic engineering

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4 Update on metabolic engineering

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6 Engineering of metabolic pathways using synthetic enzyme complexes

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17 One sentence summary.

18 The prospects for the use of synthetic enzyme complexes as a metabolic engineering tool are

19 reviewed.

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35 **Introduction**

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37 Plants provide a source of enzymes for metabolic engineering to produce valuable or useful products in
38 micro-organisms or can themselves be engineered (Andre et al., 2016; Vickery et al., 2016; Moses et al.,
39 2017). Production of high value compounds (e.g. pharmaceuticals) and nutraceuticals (e.g. omega-3 fatty
40 acids, carotenoids, tocochromanols, ascorbate, anthocyanins) involves either the introduction of novel
41 pathways into a convenient host species or optimisation of endogenous pathways. Other manipulations
42 include engineering protective secondary compound production for pest and pathogen resistance and
43 osmolytes for stress resistance. Manipulation of central metabolic pathways such as photosynthesis (e.g.
44 Calvin-Benson cycle, alternative carbon sinks, introduction of CO_2 concentrating mechanisms,
45 photorespiratory bypasses, xanthophyll cycle), starch and lipid synthesis has much potential to contribute
46 to yield improvement. The use of plants as metabolic engineering vehicles to produce valuable compounds,
47 as opposed to transferring plant pathways to microbes, will depend on feasibility and economic factors.
48 Specialised cells and tissues (glandular trichomes, resin ducts and lactifers, oilseeds) adapted to synthesise
49 and store toxic and hydrophobic compounds involved in defence may make production of certain classes of
50 compounds (e.g. isoprenoids and alkaloids) more advantageous in plants (Huchelmann et al., 2017). On the
51 other hand, plants present bottlenecks in terms of the number of genes which can be conveniently
52 manipulated and a long timeframe for optimising pathway engineering (Sweetlove et al., 2017). As an
53 alternative to stable transformation, transient expression, for example in *Nicotiana*, provides a rapid route
54 to optimising engineering and could act as a production platform (Reed and Osbourn, 2018). Also, it has
55 become apparent that cambial (stem) cells are easily cultured and produce high yields of secondary
56 compounds, such as taxol from yew (*Taxus cuspidate*) (Lee et al., 2010). This finding could lead to a
57 resurgence in the use of plant cell cultures. Recent developments in metabolic engineering and the
58 application of a synthetic biology approach have been summarised (Stewart et al., 2018). Key tools and
59 requirements for metabolic engineering in plants are a set of promoters that drive expression in specific
60 cell types, the ability to introduce multiple enzymes that are expressed at the appropriate level, targeting of
61 the pathway to specific subcellular locations/organelles and ensuring that the supply of reductant and
62 cofactors is not limiting. An example of the importance of location is illustrated by the production of
63 dhurrin in transgenic tobacco (*Nicotiana benthamiana*). Dhurrin is a cyanogenic glycoside produced by
64 sorghum and the enzymes are normally anchored to the ER. Targeting the enzymes to the thylakoid
65 membrane in a complex enables ferredoxin to be used as an alternative reductant and improves
66 performance of the pathway (Gnanasekaran et al., 2016; Henriques de Jesus et al., 2017). This example also
67 serves as an introduction to the potential of synthetic enzyme complexes to assist metabolic engineering.

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69 **The occurrence and significance of enzyme complexes: metabolons and substrate channelling**

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71 The possibility that enzymes are not randomly distributed but are associated into potentially dynamic
72 complexes consisting of enzymes in a metabolic pathway (**metabolons)** has a long history. The term
73 "metabolon" was introduced by Srere (Srere, 1985) to denote a "supramolecular complex of sequential
74 metabolic enzymes and cellular structural elements" (Srere, 1985, 1987). He proposed that metabolons
75 would enable channelling of pathway intermediates between enzymes (see the following paragraph for a
76 definition of channelling). The original definition included ribosomes and the DNA replication complex.
77 However, more recent usage excludes these highly organised structures and there is a tendency for enzyme
78 complexes to be termed metabolons in the absence of evidence for channelling or other functional
79 attributes. There are immense technical challenges in detecting potentially loose and dynamic enzyme
80 interactions (for example, by pulldowns, yeast two-hybrid and *in vivo* using fluorescent proteins) and
81 assessing their *in vivo* functionality. In plants, there are examples of enzyme associations detected by these
82 various methods and these have been reviewed (Laursen et al., 2015; Sweetlove and Fernie, 2018).
83 Examples include flavonols/isoflavonols (Achnine et al., 2004; Crosby et al., 2011; Lee et al., 2012b;
84 Dastmalchi et al., 2016; Diharce et al., 2016), polyamines (Panicot et al., 2002), sporopollenin (Lallemand et
85 al., 2013; Qin et al., 2016), alkanes (Bernard et al., 2012), indole acetic acid (Muller and Weiler, 2000;
86 Kriechbaumer et al., 2016), carotenoids (Nisar et al., 2015) and dhurrin (Moller and Conn, 1980; Laursen et
87 al., 2016). In central metabolism, the best studied examples are glycolysis and the TCA cycle (Giege et al.,
88 2003; Graham et al., 2007; Zhang et al., 2017). The glycolytic enzymes are associated with the
89 mitochondrial membrane and show dynamic behaviour; complex formation increasing with high
90 respiratory demand (Graham et al., 2007). Similarly, in mammalian cells, the purinosome, an assembly of
91 enzymes involved in purine biosynthesis, assembles when there is high demand for product (Pedley and
92 Benkovic, 2017; Baresova et al., 2018). In only a few cases has the functional significance of these enzyme
93 complexes been established. In this review, "metabolon" will be used in cases where channelling is
94 demonstrated and "enzyme complex" where two or more enzymes in a metabolic pathway are physically
95 associated.
96
97 This functional significance of metabolons has been debated but the principles are becoming clearer, not
98 least because of additional insights derived from synthetic enzyme complexes. To be effective an enzyme
99 complex must enable **channelling** (Castellana et al., 2014; Sweetlove and Fernie, 2018). Channelling is the
100 movement of an intermediate between active sites of successive enzymes with much decreased escape
101 into the bulk cytoplasmic solution (Fig. 1A). Channelling could involve direct tunnelling of intermediates
102 between active sites and/or electrostatic guidance (Elcock et al., 1997). Channelling occurs in highly-
103 organised complexes such as tryptophan synthase (Dunn et al., 2008), malate dehydrogenase/citrate
104 synthase (Bulutoglu et al., 2016) and bacterial proline oxidation. In the latter example, proline is converted
105 to glutamate *via* proline dehydrogenase (PRODH), which produces 1-pyrroline-5-carboxylate (P5C). P5C

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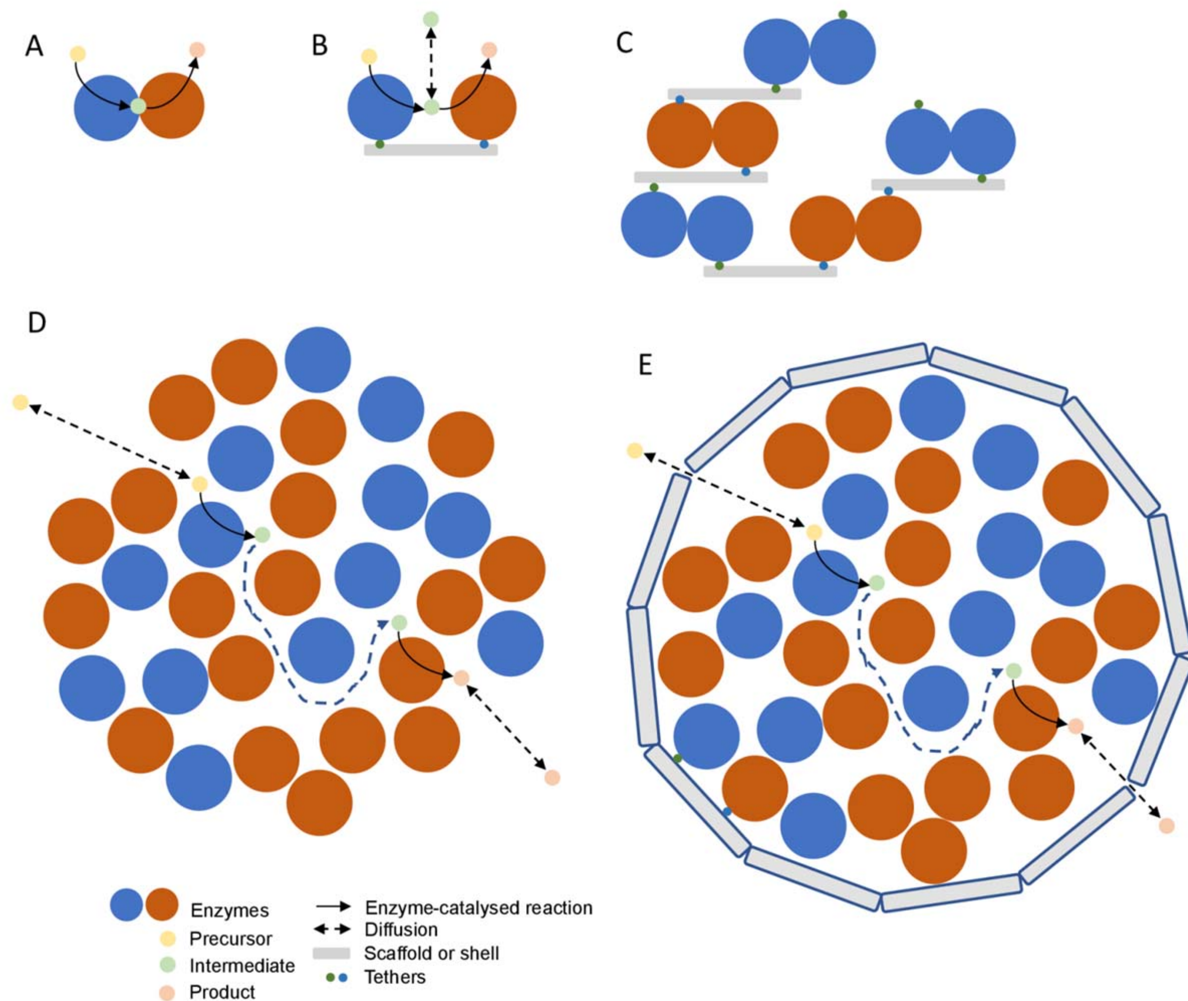

106 spontaneously hydrates to form L-glutamate-semialdehyde (GSA), which is then oxidised by P5C
107 dehydrogenase (P5CDH) to form glutamate. In many bacteria, PRODH and P5CDH comprise a bifunctional
108 enzyme and kinetic studies indicate direct channelling of P5C/GSA between the active sites. However, in
109 other cases such as *Thermus thermophilus,* the enzymes PRODH and P5CDH are on distinct proteins. Kinetic
110 studies, substrate trapping and surface plasmon resonance analysis of protein-protein interaction showed
111 orientation-dependent association between the enzymes and substrate channelling (Sanyal et al., 2015).
112 Therefore, weak but specific interactions between these enzymes have evolved to enable channelling. This
113 is an example of the Rosetta Stone hypothesis (Marcotte et al., 1999). The hypothesis suggests that if two
114 separate proteins have homologues in another genome that are located on a single polypeptide, then the
115 separate proteins are likely to interact with each other. A large proportion of the identified fusion proteins
116 are enzymes (Enright et al., 1999; Marcotte et al., 1999). From an engineering point of view, synthetic
117 fusion enzymes may or may not be effective. This is most likely because the enzymes have not co-evolved
118 complementary structures that enable effective channelling or fusion interferes with correct folding.
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120 Considering less-organised metabolons, the essential and comprehensive analysis by Sweetlove and Fernie
121 (2018) identifies the key point that the close association of sequential enzymes (in the absence of specific
122 interactions) cannot be effective at channelling because substrate diffusion rate is much faster than
123 enzyme catalysis, so the intermediate can escape (Fig. 1B). Only a few enzymes including triose phosphate
124 isomerase, carbonic anhydrase, superoxide dismutase, catalase and acetylcholine esterase operate at
125 diffusion limited rates (K_{cat}/K_m ~10^9 M^{-1} s^{-1}). Therefore, simply pairing non-coevolved enzymes will not in
126 itself be effective and, even if it is, it would increase initial rate but not steady state rate (Sweetlove and
127 Fernie, 2018). As noted above, direct channelling requires co-evolved enzymes. This is unlikely to be the
128 case when heterologous enzymes are used for engineering. Channelling requires that intermediates are not
129 in equilibrium with the bulk solvent and this could be achieved by a large cluster of enzymes, not
130 necessarily arranged in a specific manner, so that **probabilistic channelling** occurs (Castellana et al., 2014;
131 Sweetlove and Fernie, 2018). Because of localised high enzyme concentration, the probability that a
132 substrate binds to an active site before it leaves the cluster is increased and an increase in flux is also
133 predicted (Fig 1D). It is suggested that high enzyme concentration can influence the thermodynamic
134 feasibility of a pathway and its direction (Angeles-Martinez and Theodoropoulos, 2015). Evidence for
135 effective channelling in enzyme complexes *in vivo* is scarce although the wide range of central and
136 secondary metabolism pathways with interacting enzymes suggests that it is likely. Demonstration of
137 channelling is challenging, and the various approaches have been reviewed (Zhang et al., 2017; Sweetlove
138 and Fernie, 2018). Isotopic dilution is a useful technique: if channelling is occurring an added unlabelled
139 pathway intermediate will not equilibrate with the labelled
140 **[Fig 1 here]**
141 intermediate derived from a labelled precursor. Channelling has been demonstrated *in vitro* for the isolated
142 ER-bound dhurrin biosynthesis metabolon (Moller and Conn, 1980) and *in vivo* when a biosynthetic
143 complex is introduced into chloroplasts (Henriques de Jesus et al., 2017). In plants, isotope dilution
144 experiments have shown channelling in the glycolytic pathway bound to the surface of mitochondria (Giege
145 et al., 2003; Graham et al., 2007). A recent comprehensive study of the plant TCA cycle showed 158 binary
146 protein-protein interactions which were confirmed by channelling of citrate and pyruvate using isotope
147 dilution experiments (Zhang et al., 2017). This key paper provides strong evidence for physical association
148 between enzymes and the occurrence of channelling. It is also of significance since TCA cycle enzymes were
149 the first enzymes involved in the initial characterisation of metabolons (Srere, 1987; Velot et al., 1997;
150 Bulutoglu et al., 2016). The other consequences of channelling include decreasing the loss of potentially
151 reactive and toxic intermediates into the bulk solution and influencing flux at branchpoints (Zhang et al.,
152 2017; Sweetlove and Fernie, 2018). It is notable that a large proportion of metabolons are membrane-
153 associated. As well as the examples mentioned above, glycolytic enzymes associate with the cytoskeleton in
154 yeast and Arabidopsis (Araiza-Olivera et al., 2013; Garagounis et al., 2017). It is possible that channelling is
155 aided by the physical and chemical properties in the cytoplasm in proximity to surfaces such as membranes

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156 or cytoskeletal elements (Theillet et al., 2014). It is proposed that bacterial cytoplasm is divided into a
157 super-crowded “cytogel” extending 20–70 nm from the plasma membrane and more dilute cytosol (Spitzer
158 and Poolman, 2013). While not likely to influence the diffusion rate of small molecules significantly, the
159 formation of protein complexes may be favoured near surfaces, suggesting that anchoring synthetic
160 complexes to a membrane could be an advantageous strategy. Protein-protein interactions are driven by
161 several mechanisms not covered here (Williamson, 2012). A novel suggestion is that enzymes show
162 chemotactic movement along their substrate gradient which could drive their co-localisation (Wu et al.,
163 2015; Illien et al., 2017; Agudo-Canalejo et al., 2018; Zhao et al., 2018). These experiments use fluorophore-
164 tagged enzymes to follow movement in microfluidic devices but the interpretation of the fluorescence
165 correlation spectroscopy, on which the conclusions are based, has been criticised (Gunther et al., 2018).
166

167 **Construction and functioning of synthetic enzyme complexes**

168

169 The existence of enzymes complexes and the possibility that they are important in influencing metabolic
170 pathways has provided the drive to explore the use of synthetic enzyme complexes in metabolic
171 engineering. There are essentially two approaches: anchoring enzymes on scaffold molecules of various
172 kinds or encapsulating enzymes in protein coated microcompartments based on bacterial
173 microcompartments and viral capsids. Many reviews have discussed and advocated synthetic enzyme
174 complexes and possibly outnumber actual examples of its application. The reader is referred to these
175 reviews for more information (Conrado et al., 2008; Boyle and Silver, 2012; Lee et al., 2012a; Singleton et
176 al., 2014; Chessher et al., 2015; Pröschel et al., 2015; Siu et al., 2015; Polka et al., 2016; Plegaria and
177 Kerfeld, 2018; Qiu et al., 2018). A selection of examples of synthetic enzyme complexes is reviewed here in
178 relation to the methods used and outcome.
179

180 **Table 1.** Examples of metabolic pathway engineering using scaffolded enzyme complexes.
181

Product	Host organism	Approach	Outcome	Assay conditions	Reference
Ethyl acetate	*Saccharomyces cerevisiae*	Dockerin tags + cohesion/oleosin scaffold	Enzymes co-localise to lipid droplet membranes (FRET). 1.8-fold increase in product in cell lysate assay. Channelling not tested	Initial activity *in vitro*?	Lin et al., 2017
Ethanol (pyruvate decarboxylase and alcohol dehydrogenase)	*E. coli*	Filamentous scaffold proteins formed from bacterial microcompartment coat protein (PduA) fused to synthetic self-assembling coiled-coil proteins. Enzymes tagged with coiled-coil proteins. Also attached to inner membrane.	Network of cytoplasmic filaments visualised by TEM. Protein colocalization confirmed by tagged fluorescent proteins and microscopy.	Ethanol yield increased two-fold by 20 h but initial rate of increase same with or without scaffold.	Lee et al., 2018b
Dhurrin	*Nicotiana benthamiana*	Fusion of three enzymes to TatB and TatC	Dhurrin increases five-fold. Channelling	Products measured 5 d	Henriques de Jesus et al.,

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Product	Host organism	Approach	Outcome	Assay conditions	Reference
		(thylakoid membrane proteins). Transient expression, chloroplast targeted	suggested by decreased side products. Thylakoid location, but not enzyme proximity, confirmed.	post-Agrobacterium infiltration	2017
Butan-1-ol	*E. coli*	Enzymes attached to *Clostridium* exoglucanase cellulose binding domain-induced inclusion bodies *via* leucine zipper tags	Two-fold increase in butanol formation. Enzymes shown to be present in inclusion bodies.	Stable transgenic lines.	Han et al., 2017
Indole-3-acetic acid	*E. coli*	Enzymes (or split GFP) fused to DNA-binding TALE proteins assembled on a plasmid with various distances between DNA binding sites	GFP fluorescence indicates assembly on DNA scaffold. IAA production increased up to eight-fold in scaffold and binding site spacing-dependent manner	Overnight IPTG induction	Zhu et al., 2016
Methanol to fructose-6-phosphate *via* formaldehyde	*E. coli*	A multi-subunit malate dehydrogenase fused to SH3 plus a two-enzyme fusion protein with a SH3 ligand.	Assembly into a complex confirmed by TEM and dynamic light scattering. 97-fold increase in F6P *in vitro* and 2.4-fold increase in methanol consumption *in vivo*.	Faster initial MeOH consumption rate *in vivo* up to 5 h post addition.	Price et al., 2016
Indigo	*E. coli*	Bacteriophage Ø P9 and P12 proteins assembled into protein-lipid vesicles. Enzymes or fluorescent proteins fused to N-terminus of P9.	Co-localisation of fluorescent proteins and fractionation of cell extracts show assembly of lipid-protein droplets (~20 nm diameter). Indigo production increased 2.5-fold in the complex (P12-dependent)	Enzyme expression induced "overnight"	Myhrvold et al., 2016
2,3-butanediol from phosphoenolpyruvate (PK) and α-acetolactate synthase	*Saccharomyces cerevisiae*	Enzymes tagged with cohesin and dockerin to assemble *via* cohesin-dockerin interaction	Complex formation confirmed by immunoprecipitation. 1.3-fold increase in butanediol. Evidence for channelling: pyruvate produced by PK is less available for ethanol formation.	Faster product yield (g/L culture) up to 24 h after initiating a culture by dilution.	Kim et al., 2016
Branchpoint between carbamoyl phosphate (carbamoyl phosphate synthetase) utilisation for arginine (ornithine carbamoyltransferase) and carbamoyl-aspartate synthesis (aspartate carbamoyltransferase)	*E. coli*	Carbamoyl phosphate synthetase and aspartate carbamoyltransferase fusion protein expressed at high level	Increase in phase-bright cytoplasmic structures typical of protein-dense clusters. Evidence for diversion of carbamoyl phosphate away from the competing arginine synthesis pathway dependent on clustering.		Castellana et al., 2014
Alkanes (acyl-ACP-reductase and fatty aldehyde decarbonylase)	*E. coli*	Fusion protein or enzymes tagged with zinc-finger DNA binding proteins assembled on a plasmid DNA scaffold	Enzyme scaffold assembly not assessed. Fusion protein increases alkanes 4.8-fold and DNA scaffold up to 8.8-fold (dependent on enzyme stoichiometry)	24 h post-IPTG induction	Rahmana et al., 2014
Resveratrol	*Saccharomyces cerevisiae*	GBD, SH3 and PDZ combined in protein scaffolds and enzymes tagged with their ligands	Resveratrol increased by up to five-fold.	Measured 36 h (five-fold) and 96 h (two-fold) after induction	Wang and Yu, 2012
Resveratrol, 1,2-propanediol and mevalonate	*E. coli*	Enzymes tagged with zinc-finger DNA binding proteins assembled on a plasmid DNA scaffold. Random scaffold control.	Assembly shown in vitro (split YFP) and in vivo. Up to five-fold increase in product depending on enzyme proximity/pathway	24 h post induction (resveratrol and propane diol). 50 h post induction (mevalonate)	Conrado et al., 2012
Hydrogen production (ferredoxin and hydrogenase)	*E. coli*	RNA scaffolds with aptamers plus proteins tagged with aptamer binding proteins	Assembly indicated by split GFP. Mutant aptamer site controls. Up to 48-fold increase in product (dependent on scaffold geometry.	16 h after induction	Delebecque et al., 2011

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Product	Host organism	Approach	Outcome	Assay conditions	Reference
Glucaric acid	*E. coli*	GBD, SH3 and PDZ combined in protein scaffolds and enzymes tagged with their ligands	Up to five-fold increase (g/L). Enzyme stoichiometry effects observed.	48 h post induction.	Moon et al., 2010
Mevalonate	*E. coli*	GBD, SH3 and PDZ combined in protein scaffolds and enzymes tagged with their ligands	Up to 77-fold increase (g/L). Enzyme stoichiometry effects observed..	Up to 3 d post-induction	Dueber et al., 2009

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185 **Protein and protein-lipid scaffolds (Table 1).** The first report of the construction of a synthetic enzyme
186 complex in metabolic engineering was assembly of three enzymes required for synthesising mevalonic acid
187 (acetoacetyl-CoA thiolase, hydroxy-methylglutaryl-CoA synthase and hydroxymethylglutaryl-CoA reductase)
188 on a synthetic protein scaffold (Dueber et al., 2009) (Table 1). The enzymes were linked to scaffolds using
189 high affinity mammalian protein-protein interaction domains (SH3, GBD and PDZ) assembled in various
190 combinations in a synthetic scaffold protein with cognate binding domains. Each enzyme was fused to SH3,
191 GBD and PDZ ligands. Expression in *E. coli* resulted in assembly of the scaffolded proteins and an increase in
192 mevalonate accumulation when scaffolded. Following this success, further scaffolding experiments have
193 been reported (Table 1). These display an increasing diversity and ingenuity of methods used to scaffold
194 enzymes. Other high affinity protein-protein interaction domains have been harnessed (e.g. dockerin-
195 cohesin, leucine zippers, synthetic coiled-coil proteins). Generally, two to three enzymes have been
196 assembled on the scaffolds. In cases where enzymes are multimeric, attachment of one enzyme to several
197 scaffolds could allow cross linking to form larger structures (Fig. 1C). Larger conglomerations have been
198 achieved by scaffolding to very large proteins (Price et al., 2016) or to proteins liable to form inclusion
199 bodies (Han et al., 2017). A very promising approach is the production of a network of cytoskeleton-like
200 synthetic protein filaments to which enzymes are scaffolded (Lee et al., 2018a; Lee et al., 2018b). A number
201 of naturally occurring metabolons are membrane bound as noted above. In this context, enzyme complexes
202 anchored in lipid droplets have been produced by using scaffold proteins that associate with lipid-binding
203 proteins, such as oleosin (which is the coat protein for lipid droplets in oilseeds) and certain virus coat
204 proteins (Myhrvold et al., 2016; Lin et al., 2017).
205
206 **Nucleic acid scaffolds.** DNA and RNA have been explored as enzyme scaffolds (Delebecque et al., 2011;
207 Conrado et al., 2012; Rahmana et al., 2014; Zhu et al., 2016). Enzymes are tagged with DNA binding
208 proteins (e.g. zinc finger proteins, transcription activator-like effectors [TALEs]) and DNA scaffolds are
209 synthesised with specific binding site arrangements and spacing. These have been expressed in *E. coli* with
210 the scaffolds on plasmids. Plasmid copy number determines the amount of scaffold. RNA scaffolds have
211 also been tested on the basis that RNA can fold into potentially useful geometries to provide binding sites

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212 (aptamers) for tagged proteins (Delebecque et al., 2011). These approaches are appropriate for bacteria
213 but would be more problematic in plants due to the need for accessible DNA and potential RNA instability.
214
215 **Why is scaffolding successful?** The examples of scaffolding in Table 1 all show an increase in product
216 because of enzyme scaffolding, although the benefit is sometimes modest. *E. coli* and *Saccharomyces*
217 *cerevisiae* are the predominant hosts for testing scaffolds to date with the only plant example being the
218 targeting of dhurrin biosynthesis enzymes to the thylakoid membrane by transient expression in *N.*
219 *benthamiana* (Henriques de Jesus et al., 2017). Why does scaffolding work? As discussed earlier, dispersed
220 scaffolded enzyme units would be unlikely to exhibit channelling and, if they did, faster initial reaction rates
221 but not increased steady state rates would be expected (Sweetlove and Fernie, 2018). Considering the
222 examples shown in Table 1, it is generally not possible to determine if the system is at steady state because
223 many of the measurements are made hours or days after inducing scaffolding. Therefore, it is tempting to
224 propose that most of the manipulations inadvertently induce the formation of sufficiently large complexes
225 that increase local enzyme concentration, enabling probabilistic channelling and increased rate at steady
226 state (Fig. 1C). This is essentially how pyrenoids work (see below). The example of Castellana et al. (2014)
227 (Table 1) is also important because it shows that high expression of a bifunctional enzyme to form a
228 complex big enough to visualise enables channelling and diverts intermediates at a branchpoint. Controlling
229 flux at a branchpoint is also seen in the example of butanediol formation (Kim et al., 2016). Channelling was
230 demonstrated when dhurrin biosynthesis enzymes were anchored to the thylakoid membrane. The
231 pathway intermediates are reactive and when the enzymes are not anchored, LC-MS analysis detects many
232 compounds derived from them and anchoring greatly reduces their accumulation (Henriques de Jesus et
233 al., 2017). This is an important point for engineering pathways that involve reactive intermediates, where
234 the benefit could be protection against toxicity, which could be equal to the benefit of greater yield. It is
235 evident that normal metabolism causes "metabolite damage"; the production of unintended compounds
236 and it is suggested that metabolite repair enzymes could be part of the metabolic engineering tool kit (Sun
237 et al., 2017). Channelling between critical enzymes would also contribute to damage-limitation. In the
238 scaffold examples, it is likely that channelling is enabled by aggregation of the individual scaffolds into
239 larger clusters and in some of the cases this has been demonstrated (Table 1). IAA and alkane biosynthesis
240 enzymes have been detected in complexes in plants (Muller and Weiler, 2000; Bernard et al., 2012;
241 Kriechbaumer et al., 2016) but without specific evidence for channelling, so it is noteworthy that scaffolding
242 increases production of these compounds in micro-organisms (Table 1).
243
244 Most of the examples shown in Table 1 demonstrate that assembly has occurred by using techniques such
245 as co-immunoprecipitation (co-IP), fluorescent proteins (bimolecular fluorescence complementation [BiFC],
246 Förster resonance energy transfer [FRET]) and transmission electron microscopy (for larger assemblies and
247 encapsulated enzymes). Possibly, super-resolution microscopy and transmission electron cryomicroscopy

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248 (cryoEM) will be useful in providing more detailed information on the size and structure of complexes.
249 Another factor, not explicitly tackled in any of the studies, is the possibility that the scaffolding has a
250 favourable influence on the total amount of enzyme (perhaps by decreased rate of proteolysis) or
251 influences specific activity and kinetic properties. Again, the characterisation is rarely sufficiently detailed
252 to assess these possibilities. Finally, it is reasonable to suppose that unsuccessful attempts at scaffolding
253 have not been published, making it impossible to assess the probability of success.
254
255 **Synthetic microcompartments: nanoreactors**
256
257 Many bacteria produce microcompartments (**BMCs**), protein-coated nanostructures which encapsulate
258 enzymes (Fig. 1E). Their structure and functions have been well-reviewed (Kerfeld et al., 2018). They can be
259 divided into two categories by function. **Metabolosomes** contain catabolic enzymes for utilisation of carbon
260 sources *via* pathways that produce reactive intermediates. **Carboxysomes** function as part of the CO_2
261 concentrating mechanism (CCM) of photosynthetic bacteria and contain ribulose bisphosphate
262 carboxylase-oxygenase (Rubisco) and carbonic anhydrase. They occur in two distinct forms: α-
263 carboxysomes in proteobacteria and some cyanobacteria and β-carboxysomes in cyanobacteria. BMCs
264 function by concentrating enzymes (Rubisco and carbonic anhydrase) in a restricted space which enables
265 channelling as described above for other enzyme complexes. The shell presumably evolved because there is
266 an additional benefit to a diffusional barrier. Our understanding of how BMCs assemble, encapsulate the
267 correct enzymes and allow substrate and product exchange *via* pores has advanced to the point where
268 synthetic BMCs that self-assemble have been expressed in bacterial cells. Assembly of enzymes for
269 encapsulation is assisted by incorporation of encapsulation peptides (EPs) (Gonzalez-Esquer et al., 2016;
270 Plegaria and Kerfeld, 2018). In plants, there has been a focus on the possibility of introducing carboxysomes
271 into chloroplasts to mimic the cyanobacterial CCM. As in cyanobacteria, this would also need transporters
272 to concentrate bicarbonate into the chloroplast stroma. Bicarbonate would enter the carboxysomes where
273 CO_2 production is catalysed by encapsulated carbonic anhydrase and the high local concentration of
274 Rubisco drives rapid CO_2 fixation and outcompetes the oxygenase reaction (Rae et al., 2013). The first steps
275 to this goal have been achieved by successful assembly of β-carboxysome shells in chloroplasts by transient
276 expression of five shell proteins in *Nicotiana* (Lin et al., 2014). YFP tagged with a small targeting peptide
277 from the carboxysome organising protein CcmN was incorporated into the shells. In a recent breakthrough,
278 a minimal functional carboxysome was expressed in tobacco chloroplasts (Long et al., 2018). This was
279 achieved by introducing two α-carboxysome coat proteins and the large and small subunits from the
280 cyanobacterium *Cyanobium*. Chloroplasts were transformed to enable knockout of the endogenous Rubisco
281 large subunit. The resulting plants were able to grow, carrying out CO_2 assimilation with the encapsulated
282 *Cyanobium* Rubisco. The results show that this minimal carboxysome allows encapsulated Rubisco to
283 function and therefore the pores in the protein coat enable exchange of substrates and products.

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285 BMCs are related to viral capsid proteins. Capsid proteins can self-assemble in heterologous hosts and have
286 the potential to be used to encapsulate enzymes. An interesting recent example is encapsulation of an
287 indigo biosynthesis pathway from tryptophan in a virus capsid protein. The enzymes were anchored to the
288 capsid proteins using SpyTag/SpyCatcher protein fusions (Giessen and Silver, 2016). This system is based on
289 the CnaB2 domain from the fibronectin-binding protein FbaB from *Streptococcus pyogenes* and works by
290 spontaneous reaction between a lysine residue on SpyCatcher and aspartate on SpyTag to form an
291 isopeptide bond (Reddington and Howarth, 2015). Tagging the enzymes with SpyCatcher and
292 bacteriophage MS2 capsid protein with SpyTag resulted in assembly of particles in *E. coli* which increased
293 indigo production by 60 % compared to controls (Giessen and Silver, 2016). Pores of BMCs and capsids can
294 be engineered to control substrate uptake specificity (Glasgow et al., 2015). Isolated capsids showed that
295 the enzymes were markedly more stable *in vitro* because of the covalent linkages.
296

297 **Harnessing and modifying other naturally occurring structures: synthetic organelles.**

298

299 Pyrenoids and peroxisomes could be considered large enzyme complexes that enable probabilistic
300 channelling. Peroxisomes contain oxidases that produce hydrogen peroxide along with catalase, which
301 decomposes the peroxide to water. In leaves, photorespiration generates a large flux of glycolate, which is
302 oxidised in peroxisomes to produce glyoxylate (a reactive aldehyde) and hydrogen peroxide. By co-
303 operation between peroxisomes and mitochondria, photorespiration produces glycerate for recycling into
304 the Calvin-Benson cycle (Hagemann and Bauwe, 2016). Isolated spinach leaf peroxisomes produce glycerate
305 at the same rate with and without an intact membrane and, in both cases the intermediates glyoxylate and
306 hydroxypyruvate are not detected in the suspension medium (Heupel and Heldt, 1994). The results indicate
307 that the leaf peroxisome is a protein complex that maintains its integrity without the membrane boundary
308 and which exhibits channelling. More recently, interaction between glycolate oxidase and catalase was
309 shown by BiFC and co-IP (Zhang et al., 2016). The relative simplicity of peroxisomes (lack of a genome and a
310 single membrane permeable to small molecules) makes them a tempting basis for production of a synthetic
311 organelle housing engineered metabolic pathways. As noted above, the evidence for channelling in leaf
312 peroxisomes even in the absence of a membrane provides a useful starting point. Various pathways have
313 been engineered into peroxisomes, for example, to produce polyhydroxyalkanoates in Arabidopsis
314 (Mittendorf et al., 1999; Kessel-Vigelius et al., 2013). Yeast peroxisomes have been engineered to efficiently
315 produce alkanes and fatty alcohols from acyl-CoAs, with evidence that the high enzyme concentrations
316 enabled channelling (Zhou et al., 2016). These pathways use the acyl-CoA metabolising capacity of
317 peroxisomes. Modification of the existing peroxisomal protein import system can increase its efficiency for
318 importing enzymes (DeLoache et al., 2016). However, the recent creation of a novel protein import system
319 which runs in parallel with the endogenous system provides a step towards synthetic peroxisomes (Cross et

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320 al., 2017). Deeper understanding of proliferation mechanisms and the protein-protein interactions that
321 hold the peroxisomal matrix together will also assist in reaching this goal.
322
323 The pyrenoid could provide another starting point for producing a protein aggregate with high enzyme
324 concentration that enables channelling. This structure consists of an aggregate of Rubisco in the
325 chloroplasts of algae and some liverworts and is required for their CO_2 concentrating mechanism (CCM). It
326 traps CO_2 produced by carbonic anhydrase allowing improved Rubisco activity (Meyer et al., 2017; Küken et
327 al., 2018). The protein components of this structure have been identified and the protein EPYC1, present in
328 high concentration, interacts with Rubisco, forming a scaffold (Mackinder et al., 2016; Mackinder et al.,
329 2017). Additionally, the resulting structure is liquid, rather than crystalline, and undergoes a phase
330 transition and fission during cell division (Freeman Rosenzweig et al., 2017). These structures suggest the
331 possibility of making synthetic organelle-like structures without walls or membranes for metabolic
332 engineering and, of course, the introduction of pyrenoids into plant chloroplasts is a potential route for
333 improving photosynthesis (Mackinder, 2018).
334
335 **Conclusion**
336 It is evident that the amount of final product in engineered metabolic pathways can be increased by various
337 ingenious scaffolding approaches. The most likely explanation is that the resulting enzyme aggregates are
338 (often inadvertently) large enough to enable probabilistic channelling due to increased local enzyme
339 concentration. Pyrenoids work in the same manner, while encapsulation in BMC and capsid coat proteins
340 increases enzyme concentration and provides an additional (potentially selective) diffusion barrier. Leaf
341 peroxisomes are robust protein complexes that can hold together without their membrane and exhibit
342 channelling. Peroxisomes and pyrenoids could form the basis for engineering multienzyme metabolic
343 pathways which benefit from channelling. Ultimately, the widespread use of channelling in plant metabolic
344 engineering will be determined by a balance between the extra time required to introduce and optimise
345 enzyme assemblies *versus* the potentially modest benefit in product yield. Channelling could provide a
346 critical advantage if it enables production of compounds with highly reactive and toxic pathway
347 intermediates or improves diversion of central metabolism intermediates into the engineered pathway.
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372
373 **Accession Numbers**
374
375 **Tables**
376
377 **Table 1.** Examples of metabolic pathway engineering using scaffolded enzyme complexes.
378
379
380
381 **Figure Legends**

382 **Figure 1.** Enzyme assemblies and their influence on substrate channelling. **A**. Two closely associated ("co-
383 evolved") enzymes enabling direct channelling of the intermediate between active sites. The active sites
384 could be located on separate proteins or on a single bifunctional protein. **B**. Tagged enzymes attached to a
385 synthetic scaffold protein, nucleic acid or lipid scaffold (see Table 1). There is little channelling since the
386 diffusion rate of the intermediate is much faster than enzyme activity. **C**. The same assembly as (B) but
387 showing how multimeric scaffolded enzymes can form larger aggregates of high enzyme concentration. **D**.
388 A large assembly of enzymes providing high local enzyme concentration enables probabilistic channelling.
389 Here, the high enzyme concentration increases the chance that the intermediate binds to an enzyme active
390 site before diffusing away. **E**. An encapsulated enzyme assembly is identical to (D), but a self-assembling
391 protein coat provides an additional diffusion barrier with pores at the vertices to allow (selective) exchange
392 of substrates and products. The enzymes could be tethered to the coat proteins. Examples are bacterial
393 microcompartments (BMCs) specialised for utilisation of carbon sources in pathways involving reactive
394 intermediates (metabolosomes) and for CO_2 fixation with encapsulated carbonic anhydrase and Rubisco
395 (carboxysomes). Eukaryotes lack BMCs but have pyrenoids (an aggregation of Rubisco surrounded by a
396 loose starch sheath found in algae and hornworts) and peroxisomes. Peroxisomes house enzymes that
397 produce toxic products and could be considered analogous to metabolosomes. They are bounded by a
398 membrane that is relatively permeable to small molecules and exhibit channelling.
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ADVANCES

- Multiple physical interactions between plant TCA cycle enzymes have been detected, which enables channeling of intermediates at some steps. This finding confirms the functional significance of TCA cycle metabolons, which are conserved across many species.
- A functional streamlined α-carboxysome encapsulating Rubisco has been introduced into tobacco chloroplasts, providing a significant step in engineering a synthetic carbon dioxide-concentrating mechanism into C_3 plants.
- Pyrenoids, an aggregation of Rubisco in algal chloroplasts forming their carbon dioxide concentrating mechanism (CCM), are dynamic liquid-like structures and a novel protein EPYC1 is involved in their structure. This discovery paves the way for designing synthetic CO_2-concentrating mechanisms and could form the basis for engineering pyrenoid-like synthetic enzyme complexes for other purposes.
- Anchoring a complex of dhurrin (a cyanogenic glycoside) biosynthesis enzymes to the thylakoid membrane in tobacco enables use of ferredoxin as an alternative reductant and improves pathway yield by channeling and decreasing the escape of reactive pathway intermediates.

OUTSTANDING QUESTIONS

- How many of the enzyme complexes detected in plants by protein-protein interaction exhibit channeling with potential advantages for flux, control of branch points or sequestration of reactive intermediates?
- Can increased knowledge of pyrenoid structure be harnessed to enable their use in metabolic engineering beyond photosynthesis?
- Can peroxisomes be re-purposed to allow channeling in introduced pathways?
- Innovative techniques for constructing synthetic enzyme complexes are so far almost entirely restricted to micro-organisms. Given the relatively small gain in product formation, will there be situations where investing in the optimization of channeling in plants would be beneficial?
- Could the high throughput possible in micro-organisms enable forced evolution of multiple enzymes to optimize their interactions before transfer to plants?

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