

1 **Synthetic metabolons for metabolic engineering**

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25 **Abstract**

26 It has been proposed that enzymes can associate into complexes (metabolons) that increase
27 the efficiency of metabolic pathways by channelling substrates between enzymes. Metabolons
28 may increase flux by increasing local concentration of intermediates, decreasing the
29 concentration of enzymes needed to maintain a given flux, directing the products of a pathway
30 to a specific subcellular location or minimise the escape of reactive intermediates. Metabolons
31 can be formed by relatively loose non-covalent protein-protein interaction, anchorage to
32 membranes and (in bacteria) by encapsulation of enzymes in protein-coated
33 microcompartments. Evidence that non-coated metabolons are effective at channelling
34 substrates is scarce and difficult to obtain. In plants there is strong evidence that small
35 proportions of glycolytic enzymes are associated with the outside of mitochondria and are
36 effective in substrate channelling. More recently, synthetic metabolons, in which enzymes are
37 scaffolded to synthetic proteins or nucleic acids, have been expressed in micro-organisms and
38 these provide evidence that scaffolded enzymes are more effective than free enzymes for
39 metabolic engineering. This provides experimental evidence that metabolons may have a
40 general advantage and opens the way to improving the outcome of metabolic engineering in
41 plants by including synthetic metabolons in the toolbox.

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43 **Key words:** Bacterial microcompartments, cyanogenic glycosides, flavonoids, metabolic
44 engineering, photosynthesis

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49 **Introduction**

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51 The concept of “synthetic biology” embraces the idea of redesigning organisms from the
52 ground up. It is enabled by the standardisation of DNA parts and experimental procedures that
53 allows automation, reliable output measurements and robust biological engineering; the
54 thorough characterisation of DNA parts so that their behaviour in a given context is
55 predictable; and the further modularisation of these parts into devices that perform well-
56 defined functions. This latter concept allows further abstraction in which this information (e.g.
57 DNA sequence, part or device data) is hidden thereby reducing the observable details allowing
58 the researcher to focus on a few key concepts at a time (Freemont and Kitney, 2012). As an
59 example, selectable marker cassettes may be considered devices - nowadays it is unusual for
60 the researcher to look in detail at what resides within these markers, we know they confer
61 resistance to a given antibiotic or herbicide and for most of the time that is all that is required.
62 In applying this approach across the board the parts and devices can be used and re-used to
63 provide predictable, highly controlled levels of gene expression to produce regulatory circuits
64 or novel metabolic pathways with predictable outcomes. This approach often requires
65 transformation with multiple genes and most of the examples are currently from readily
66 transformed microorganisms. The effort is aided by improved gene cloning technology and the
67 decreasing cost of synthesising genes *de novo*. In bacteria, the ability to produce novel
68 organisms with streamlined genomes, in which the various patches and fixes installed during
69 evolution are replaced or streamlined, has emerged (Gibson *et al.*, 2010) and will allow
70 production of more efficient vehicles for production of useful end products. This review will
71 not address the nuts and bolts of DNA manipulation and the problems of expressing multiple
72 transgenes in plants at controlled levels in specific cell types but will rather focus on proteins

73 and enzymes and how these might be designed and manipulated more effectively for plant
74 metabolic engineering. Metabolic engineering is well established in microorganisms and this is
75 where synthetic biology approaches first emerged. There are metabolic engineering success
76 stories in plants for example expression of carotene biosynthesis genes in rice grain to create
77 “golden rice”, multi-vitamin corn (Naqvi *et al.*, 2009) and altered seed lipids (DellaPenna, 2001;
78 Mayer *et al.*, 2008; Napier and Graham, 2010; Ruiz-Lopez *et al.*, 2014; Ye *et al.*, 2000).
79 Metabolic engineering could also be employed to improve crop yield by manipulating
80 photosynthesis, starch synthesis and production of osmolytes and defensive compounds.
81 However, the result of over-expression of a few judiciously chosen enzymes is often
82 disappointing due to lack of understanding of metabolic regulation and possible toxicity of end
83 products. Therefore use of approaches that go beyond enzyme over-expression should be
84 considered. Improved photosynthesis, nutrient use and stress resistance would not only
85 increase the efficiency of plant production but may also allow for the use of plants as chemical
86 factories. Such synthetic biology approaches are emerging in microbial metabolic engineering
87 and the purpose of this review is to consider one of these approaches- the creation of synthetic
88 enzyme complexes (metabolons) and assess their potential application in plants.

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90 **Metabolons, microcompartments and metabolic channelling**

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92 The term “metabolon” was coined by Srere to describe complexes of enzymes that carry out
93 sequential reactions (Srere, 1987, 2000). This definition is potentially wide and could include
94 stable assemblies of enzymes that carry out complex or vectorial series of reactions such as
95 ribosomes (protein synthesis), proteasomes (protein degradation), photosynthetic and
96 respiratory electron transport complexes, ATP synthase, mammalian and fungal fatty acid

97 synthase, polyketide synthases, non-ribosomal peptide synthases and cellulosomes (cellulose
98 degradation complexes in bacteria) (Hyeon *et al.*, 2013). These multi-protein complexes carry
99 out series of reactions that require intermediates to be shepherded (channelled) precisely. The
100 relatively complex series of reactions required for fatty acid synthesis are located in a dimeric
101 multifunctional fatty acid synthase in mammals and fungi (Voet and Voet, 2004). Similarly,
102 polyketide synthases and non-ribosomal peptide synthases consist of several proteins each
103 with multiple active sites, allowing synthesis of complex molecules such as antibiotics (Khosla
104 *et al.*, 1999). In the case of the pyruvate dehydrogenase complex, tryptophan synthase and
105 carbamoyl phosphate synthetase, intermediates are effectively channelled between active
106 sites (Voet and Voet, 2004). Apart from these examples of stable or close complexes, where
107 intermediates are directly shuttled between active sites, the existence of functional
108 metabolons based on loose or transitory enzyme-enzyme interactions is a matter of
109 controversy. The proposed advantages of metabolons include increasing the concentration of
110 intermediates at the active sites of sequential enzymes and minimizing escape of reactive or
111 potentially toxic intermediates (Jørgensen *et al.*, 2005; Moller, 2010; Srere, 1987; Sweetlove
112 and Fernie, 2013; Winkel, 2004). These possibilities are illustrated in the review by Chen and
113 Silver (2012). The general argument against metabolons providing an advantage is that
114 metabolites diffuse too quickly relative to reaction rates and cell size for there to be a
115 significant concentration of intermediates in the vicinity of the complex. While demonstrating
116 that complexes are likely to occur *in vitro* is relatively easy, assessing their functionality is much
117 more difficult. To assess the significance of proposed complexes based on protein-protein
118 interaction methods such as pull-downs (e.g. TAP-tagging), surface plasmon resonance and
119 yeast2-hybrid assays, it is necessary to demonstrate that association occurs *in vivo*. This is
120 probably best done with fluorescently-tagged proteins using techniques such as Förster

121 resonance energy transfer (FRET) and bimolecular fluorescence complementation (BIFC) (Ohad
122 *et al.*, 2007). However, methods with a higher throughput such as yeast 2-hybrid or pull-downs
123 followed by protein identification using mass spectrometry are ideal for initial screening. There
124 are numerous examples of proposed metabolons in animals and microorganisms (Chen and
125 Silver, 2012; Srere, 1987). Recent examples include glycolysis (Araiza-Olivera *et al.*, 2013;
126 Puchulu-Campanella *et al.*, 2013), the TCA cycle (Meyer *et al.*, 2011; Mitchell, 1996; Velot *et al.*,
127 1997), amino acid biosynthesis (de Cima *et al.*, 2012; Hutson *et al.*, 2011; Islam *et al.*, 2007),
128 acyl ester biosynthesis (Jiang and Napoli, 2013) and melanin biosynthesis (Sugumaran *et al.*,
129 2000). While most of the examples involve protein-protein interactions (with the possible
130 inclusion of a membrane-located anchoring proteins), other methods of complex formation
131 have been identified, for example association of glycolytic enzymes with F-actin (Araiza-Olivera
132 *et al.*, 2013) or targeting of enzymes to lipid droplets (Jiang and Napoli, 2013). In plants,
133 plastoglobuli – lipid inclusions in chloroplasts- have enzymes of isoprenoid biosynthesis and
134 chlorophyll degradation associated with them (Lundquist *et al.*, 2012; Nacir and Bréhélin, 2013)
135 and so could organise enzymes that metabolise lipophilic substrates. Despite the relatively
136 abundant evidence for physical association of enzymes into metabolons, more definitive
137 evidence that pathway intermediates are effectively channelled (for example from isotope
138 dilution studies) is very rare (Graham *et al.*, 2007), leading to scepticism about their function.
139
140 Given the increasing number of metabolons proposed in other organisms, it is not surprising
141 that the evidence for association of enzymes into complexes in plants is accumulating. A
142 number of reviews cover this evidence (Jørgensen *et al.*, 2005; Sweetlove and Fernie, 2013;
143 Winkel, 2004). Pathways, or parts of pathways, in which enzymes are associated in complexes
144 include glycolysis and various biosynthesis pathways (polyamines, flavonoids,

145 phenylpropanoids, cyanogenic glycosides, sporopollenin, long chain alkanes, cyanogenic
146 glycosides and indole acetic acid). The references and evidence for the existence of enzyme
147 complexes and their functional significance are shown in Table 1 while other examples of
148 possible secondary metabolite metabolons have been reviewed elsewhere (Jørgensen *et al.*,
149 2005). Mining the high throughput Arabidopsis yeast 2-hybrid data should indicate further
150 pathways in which enzymes form complexes (Arabidopsis Interactome Mapping Consortium,
151 2011; Zhang *et al.*, 2010). It is possible that the search for metabolons in plants could also be
152 guided by the conservation of protein-protein interactions across phylogenetic groups. Clearly,
153 the demonstration of enzyme complexes does not prove that pathway flux is improved or that
154 potentially toxic intermediates are confined. Evidence that this benefit occurs for some
155 pathways was discussed in the previous section. In plants, this level of evidence is sparse. In
156 the case of association of glycolytic enzymes with the outer mitochondrial membrane, isotope
157 dilution experiments have shown that addition of unlabelled intermediate has little effect on
158 the specific activity of products produced from a labelled precursor (Giege *et al.*, 2003; Graham
159 *et al.*, 2007). This is key evidence that indicates not only are the enzymes spatially organised,
160 but that there is also a degree of direct substrate channelling between them. In the case of
161 glycolysis, it is important to note that only a small proportion of the enzymes locate to the
162 mitochondria and therefore the effect is local.

163

164 The idea that enzymes of the Calvin-Benson cycle may also aggregate into metabolons has
165 been around for some time (Anderson *et al.*, 2006) but their existence remains unproven. The
166 Calvin-Benson cycle however does provide an alternative paradigm for regulation of central
167 metabolism by protein complexes. A well-documented multi-enzyme complex exists between
168 two, non-sequential Calvin-Benson cycle enzymes, phosphoribulokinase (PRK) and

169 glyceraldehyde 3-phosphate dehydrogenase (GAPDH) that is mediated by the small chloroplast
170 protein CP12 (Wedel and Soll, 1998; Wedel *et al.*, 1997). When PRK and GAPDH are aggregated
171 within this complex they are far less active and more susceptible to allosteric inhibition than
172 their unbound forms. The PRK/GAPDH/CP12 complex, which is present in darkened leaves, is
173 capable of almost instantaneous dissociation (activation) upon illumination, and similar, rapid
174 association (deactivation) upon transfer to darkness (Howard *et al.*, 2008). This process,
175 dependent upon the activity of the photosynthetic electron transport chain and mediated by
176 thioredoxin (Howard *et al.*, 2008; Marri *et al.*, 2009) allows swift, dynamic
177 activation/inactivation of enzyme activity in response to environmental changes. This
178 mechanism provides an alternative function for synthetic metabolic protein complexes in line
179 with the aims of making smarter metabolically engineered pathways that are responsive to
180 cellular demands (Zhang *et al.*, 2012). Despite the lack of strong evidence for a more general
181 Calvin-Benson cycle metabolons, recent detailed flux analysis using $^{13}\text{CO}_2$ labelling in
182 *Arabidopsis thaliana* provides evidence for discrete pools of metabolites, a feature that could
183 indicate some degree of metabolite channelling within the stroma (Szecowka *et al.*, 2013).

184

185 Bacterial microcompartments (metabolosomes/enterosomes) consist of enzymes that are
186 encapsulated in self-assembling protein coats forming particles that are of the order of 100 nm
187 in diameter. They are found in various bacteria and generally contain pathways that have
188 reactive or toxic intermediates, for example in ethanol, ethanolamine and propanediol
189 utilisation (Kerfeld *et al.*, 2010). Cyanobacterial carboxysomes, which are part of the carbon
190 dioxide concentrating mechanism of these organisms, are a well-known example (Kerfeld *et al.*,
191 2010; Rae *et al.*, 2013). They contain ribulose bisphosphate carboxylase-oxygenase (RuBisCO)
192 and carbonic anhydrase (CA). The coat proteins form suitably charged pores that allow ingress

193 of substrates (RuBP and bicarbonate). Within the carboxysome, CO₂ release from bicarbonate
194 is catalysed by CA and then RuBisCO catalyses the carboxylation of RuBP to form
195 phosphoglyceric acid, the first product of the Calvin-Benson cycle. The localised release of CO₂
196 in the carboxysome outcompetes the oxygenase reaction of RuBisCO which otherwise results
197 in “wasteful” photorespiration (Bauwe *et al.*, 2012; Maurino and Peterhansel, 2010). Protein-
198 coated microcompartments could therefore provide another route to controlling or improving
199 metabolic flux and their introduction into C₃ chloroplasts, along with bicarbonate transporters
200 has been suggested as an approach to decreasing photorespiration (Price *et al.*, 2013).

201

202 **Synthetic metabolons**

203 The previous discussion indicates that while a number of metabolons have been proposed, the
204 evidence that they function in improving metabolic flux is scarce. Perhaps the most direct way
205 to determine the utility of metabolons is to introduce them into cells and assess their effect on
206 flux or product formation. The possibility of constructing synthetic enzymes complexes has
207 been discussed in a number of recent reviews (Boyle and Silver, 2012; Lee *et al.*, 2012). While
208 there are a number of ways that synthetic enzyme complexes could be assembled, the first
209 approach to have been employed is to assemble enzymes onto a synthetic scaffold protein
210 using protein interaction domains. This makes use of the reasonably high affinity and specific
211 interactions between protein interaction modules and their peptide ligands derived from
212 various scaffolding and signalling systems. A gene encoding a synthetic scaffold protein
213 containing the protein interacting domains separated by spacer sequences in the required
214 order and stoichiometry is synthesised. The enzymes are tagged with the corresponding
215 peptide ligands. This approach has been used to scaffold three enzymes of the mevalonic acid
216 biosynthesis pathway in *E. coli* (Dueber *et al.*, 2009). A synthetic scaffold protein was

217 constructed carrying three high affinity protein interaction domains: the Src homology domain
218 3 (SH3) from the mouse Crk adapter protein (Wu *et al.*, 1995) the PDZ domain from the α -
219 syntrophin protein from mouse (Schultz *et al.*, 1998) and the GTPase-binding domain (GBD)
220 from the neuronal Wiskott-Aldrich syndrome protein (N-WASP) (Kim *et al.*, 2000). The three
221 enzymes were tagged with their cognate peptide ligands. When expressed together, the
222 ligands reversibly bind to the scaffold protein and form a complex. Scaffolding increased
223 product titre 77-fold compared to unscaffolded enzymes- and with lower overall protein
224 expression (HMG-CoA intermediate is toxic) (Dueber *et al.*). Similar approaches with a 3 step
225 biosynthesis pathway for glucaric acid from glucose in *E. coli* yielded a 5 fold increase in yield of
226 product (Moon *et al.*, 2010). Similarly, in yeast, scaffolding 4-coumarate:CoA ligase and
227 stilbene synthase increased the yield of resveratrol by 5-fold compared to unscaffolded
228 enzymes (Wang and Yu, 2012). For this two enzyme pathway, a fusion protein was also tested
229 but was less effective than scaffolded enzymes perhaps because of folding problems (Zhang *et*
230 *al.*, 2006).

231

232 Scaffolding has also been shown to work *in vitro*. In this case, triose phosphate isomerase,
233 aldolase and fructose 1,6-bisphosphatase were scaffolded *in vitro* resulting in a 38 to 48-fold
234 increase in reaction rate for scaffolds free in solution or bound to cellulose respectively (You
235 and Percival Zhang, 2013). This example used a system based on the cellulosome complex from
236 various cellulose degrading bacteria. The cellulosome consists of a scaffoldin protein containing
237 dockerin domains to which proteins containing cohesin domains bind. In this example, the
238 enzymes were tagged with three dockerin domains and the scaffold was constructed from the
239 three respective cohesin domains with an N-terminal carbohydrate binding domain. The three
240 dockerin/cohesin domains originated from three different cellulolytic microorganisms.

241

242 The factors underpinning increased product titre of these synthetic scaffold complexes have
243 been investigated. Variation in linker length and protein positioning affect yield and support
244 the conclusion that increased enzyme proximity is a key factor (Lee *et al.*, 2012). Also, it is
245 evident that enzyme stoichiometry within the metabolons is important, revealing an issue for
246 conventional metabolic engineering where enzyme over-expression levels are often not
247 precisely controlled. Although the experiments with synthetic metabolons reveal an
248 advantage, the explanation for this is not obvious. It has been argued that the product of an
249 enzyme is likely to diffuse away very quickly along its concentration gradient because typical
250 enzyme reaction rates are much slower than diffusion (Sweetlove and Fernie, 2013) so that
251 proximity might not provide significantly increased substrate concentration. The actual
252 diffusion rates in the cytoplasm are therefore important. Evidence from the mobility of
253 fluorescently labelled glucose in *E. coli* cells suggests that it diffuses ~10 times more slowly *in*
254 *vivo* ($50 \mu\text{m}^2 \text{s}^{-1}$) than in water ($400 \mu\text{m}^2 \text{s}^{-1}$), reflecting the intense macromolecular crowding
255 of the cytoplasm (Mika *et al.*, 2010). Proteins, being larger, diffuse more slowly: $3\text{-}10 \mu\text{m}^2 \text{s}^{-1}$ in
256 the cytoplasm. Evidence for the existence of diffusion gradients would be difficult to produce if
257 enzymes are evenly distributed in the cytoplasm but examples where a small molecule is
258 produced in a localised manner suggest that concentration gradients can exist. Firstly, it is
259 well-known that localised release of Ca^{2+} within cells leads to localised high concentration
260 microdomains (Berridge, 2006). Secondly, hydrogen peroxide is produced locally in some cells
261 by plasma membrane NADPH oxidase, an example being growing pollen tubes where a
262 gradient of hydrogen peroxide forms. Visualised by a ROS sensitive fluorescent probe,
263 hydrogen peroxide concentration is highest at the tip (Potocký *et al.*, 2007). Therefore, relative
264 to the rate of production and breakdown, diffusion is apparently not fast enough to equilibrate

265 hydrogen peroxide concentration throughout the cell. It is also likely that the diffusion rate of
266 small molecules will depend on their chemical properties (for example propensity to interact
267 with proteins) as well as size. Interestingly, in a modification of the *in vitro* scaffolding of
268 triose phosphate isomerase, aldolase and fructose 1,6-bisphosphatase, replacement of the
269 original aldolase with another of lower specific activity removed the advantage of scaffolding
270 (You and Zhang, 2013). This observation shows that the specifics of enzyme kinetics are
271 critical. Modelling of the system is needed to clarify the important factors, particularly to
272 determine if increased local substrate concentration is generally important. Because of
273 relatively rapid diffusion, the size of the complex may also be critical: bigger complexes are
274 predicted to capture and use more of the intermediates (Lee *et al.*, 2012). These authors
275 suggested 100 nm would be ideal for the scaffolded mevalonate pathway. In this system, since
276 some of the enzymes are multimeric, they could bind to multiple scaffolds producing a larger
277 complex. Another potentially important effect could be control of pathway branch points and
278 competition between different pathways for the same intermediates. In the latter case, a
279 higher proportion of the product of a scaffolded enzyme might be passed on to its neighbour
280 than to an unscaffolded competitor. Scaffolding also provides a potential means to control
281 branch points by manipulating protein-protein interactions by signal molecules. As an example,
282 one can envisage that binding sites for pathway products or precursors could be included in a
283 synthetic scaffold such that binding causes conformational changes that alter the distance
284 between the scaffolded enzymes or cause dissociation of the complex. This would be
285 analogous to FRET-based glucose sensors (Jones *et al.*, 2013). There is already evidence that
286 metabolic status can affect the conformation of the ER-bound flavonoid biosynthesis
287 metabolons in plants, potentially directing intermediates down one branch of the pathway or
288 another (Crosby *et al.*, 2011). It is very unlikely that any of the current examples have

289 introduced the more extreme form of substrate channelling shown by enzyme complexes such
290 as tryptophan synthetase where intermediates pass through tunnels lined with amino acid
291 residues of appropriate charge and never enter the external water. Introduction of direct
292 channelling would require more sophisticated protein engineering to create and orientate
293 channels. These examples provide evidence that synthetic scaffolds can improve flux and
294 product formation even though the mechanisms are not entirely clear and even counter-
295 intuitive given the fast diffusion rates of small molecules (Lee *et al.*, 2012; Sweetlove and
296 Fernie, 2013). Nevertheless, the results provide a rationale for the natural occurrence of
297 metabolons.

298

299 Since proteins can bind to nucleic acids with high affinity and in a nucleotide sequence-specific
300 manner (e.g. transcription factors), they could also act as scaffolds for enzymes. This has been
301 demonstrated in *E. coli* for introduced resveratrol, 1,2-propanediol and mevalonic acid
302 biosynthetic pathways (Conrado *et al.*, 2012). In this example, each enzyme was fused with a
303 DNA binding zinc finger domain. These were expressed in *E. coli* along with a plasmid
304 containing multiple copies of each Zn finger binding nucleotide sequence. Evidence that the
305 enzymes assembled on the plasmid was obtained and, in each case, product formation was
306 increased over a random scaffold control. Interestingly, the increase in mevalonic acid was
307 much less than the 77-fold reported for protein scaffolds (Dueber *et al.*, 2009). Problems with
308 plasmids will include sufficient copy number to scaffold available protein and supercoiling. This
309 method is currently not applicable to plants but assembly on RNA scaffolds is a possibility. RNA
310 molecules (aptamers) that fold into specific conformations and with sequences that can
311 specifically bind small molecules and proteins could be used as scaffolds (Delebecque *et al.*,
312 2011). Application of this approach to hydrogen production in *E. coli* using ferredoxin and

313 hydrogenase tagged with adapter proteins that bind to specific RNA aptamers resulted in
314 stable scaffolded protein/RNA complexes and increased the rate of hydrogen production by up
315 to 48 fold compared to unscaffolded. Clearly, in this case, electron transfer from reduced
316 ferredoxin to hydrogenase requires very close proximity, but as with protein scaffolds, there is
317 no reason to suppose this approach would not work for other pathways. A potential problem
318 with expression of RNA in sufficient amounts is stability. However, advantages are the complex
319 3D structures that RNA can form and the possibility of designing specific protein binding
320 aptamers. This could extend to the possibility of adding riboswitch-like control mechanisms in
321 which small molecules also bind to the RNA scaffold and change its conformation. This could
322 change the distance between scaffolded enzymes and modulate flux according to the
323 concentration of an “allosteric” controlling molecule.

324

325 Bacterial microcompartments (BMCs) provide inspiration for another approach to creating
326 synthetic metabolons in which enzymes are encapsulated in self assembling protein shells
327 (Frank *et al.*, 2013; Retterer and Simpson, 2012). This provides a physical diffusion barrier to
328 pathway intermediates thus improving local concentration of intermediates, but has the
329 requirement that the shell proteins provide pores for substrate to enter and product to leave.
330 Although the structure of some BMCs is complex, recent work shows that they can be
331 assembled in host bacteria and protein cargo can be encapsulated. Thus, the shell proteins
332 from the propanediol utilization BMC from *Citrobacter freundii* can be expressed in *E. coli* to
333 form empty structures (Parsons *et al.*, 2010). Expression of a carboxysome operon from
334 *Halothiobacillus neapolitanus*, including pore protein, coat protein, CA and Rubisco in *E. coli*
335 produced functional carboxysomes (Bonacci *et al.*, 2012). Significantly, the *Salmonella enterica*
336 ethanolamine utilization (*eut*) shell protein expressed in *E. coli* assembles to form a polyhedral

337 structure. These structures could be loaded with enhanced green fluorescent protein fused to
338 an appropriate N-terminal signal sequence (Choudhary *et al.*, 2012). Therefore, in principle, it
339 should be possible to encapsulate multiple enzymes in synthetic BMCs. Utility in metabolic
340 engineering will depend not only on successful assembly and targeting of enzymes but also on
341 understanding how to include entry and exit pores for substrate and product. Clearly, the way
342 is open to attempt assembly of microcompartments in plant cells and the introduction of
343 carboxysome-like compartments into chloroplasts to improve carboxylation efficiency is a
344 potential target. Viral coat proteins offer another source of self assembling
345 microcompartments. Empty cowpea mosaic virus particle (termed empty virus-like particles,
346 eVLPs) can be assembled in plants by expressing the virus coat precursor protein and a
347 protease that cleaves it into the final form (Saunders and Lomonosoff, 2013). Other
348 approaches to assembling synthetic metabolons could include targeting enzymes to the
349 cytoskeleton (Araiza-Olivera *et al.*, 2013) or using membrane anchored proteins as in the
350 naturally-occurring flavonoid and glycolytic metabolons (Crosby *et al.*, 2011; Graham *et al.*,
351 2007) that tether the pathway enzymes. In this way metabolons could be targeted to specific
352 membranes perhaps directing transport of products into specific subcellular compartments
353 (e.g. the vacuole for storage of potentially toxic products). Positioning might also allow a
354 pathway to efficiently access substrates emanating from an organelle.

355 Microcompartmentation and localisation of signalling complexes is achieved by anchoring
356 complexes to lipid rafts. These are specialised membrane microdomains that are rich in
357 cholesterol and sphingolipids into which GPI-anchored proteins insert thereby fostering close
358 proximity of proteins (Simons and Ikonen, 1997). The NADPH oxidase (NOX) complex which
359 includes NOX and various regulatory proteins that control the oxidative burst is an example
360 from animals (Shao *et al.*, 2003) that may also operate in plants (Borner *et al.*, 2003).

361

362 **Looking to the future: synthetic metabolons in plants.**

363

364 The potential advantages of metabolons and microcompartments include increased flux (or
365 less enzyme needed to maintain a given flux), containment of potentially toxic or biologically
366 active intermediates, control over enzyme stoichiometry and the ability to control
367 branchpoints and thereby direct metabolism. Based on the experience of the introduction of
368 metabolons into microorganisms, it is clear that a similar strategy should be considered as part
369 of the plant metabolic engineering toolbox. Since secondary metabolism provides clear
370 examples of enzyme complexes (Jørgensen *et al.*, 2005) it is likely that metabolons will be of
371 particular value in production of exotic compounds in plants. Pathways that have exotic or
372 reactive intermediates may benefit from channelling. In the future, metabolons could
373 incorporate switching mechanisms as proposed for flavonoid biosynthesis (Crosby *et al.*, 2011)
374 in which metabolic status is sensed causing association or disassociation of the complexes
375 (Michener *et al.*, 2012). It is interesting that, as well as associating into complexes, the enzymes
376 of the flavonoid biosynthesis pathway are under tight transcriptional control suggesting that
377 control of enzyme concentrations well as complex formation is important in this pathway. The
378 appearance of an array of genetically-encoded fluorescent metabolite sensors also opens the
379 way to monitoring metabolism in cells in “real time” and, in conjunction with flux analysis using
380 labelled substrates, will allow improved understanding of metabolic control and bottlenecks
381 (Berg *et al.*, 2009; Hung *et al.*, 2011; Jones *et al.*, 2013; Michener *et al.*, 2012; Tantama *et al.*,
382 2011).

383

384 The cyanobacterial carboxysome (and algal pyrenoids) are archetypal metabolons which bring
385 about CO₂ concentration at the active site of RuBisCO by bringing carbonic anhydrase (CA) and
386 RuBisCO into close proximity along with a supply of bicarbonate. Therefore, one possible way
387 to improve photosynthesis would be to introduce mechanisms inspired by these systems into
388 C₃ crop plants (Price *et al.*, 2013). This approach could include introduction of bicarbonate
389 pumps into the chloroplast envelope and arrangement of RuBisCO and CA into scaffolded
390 complexes or even into synthetic carboxysomes. The latter approach should be possible given
391 the recent demonstrations that simple microcompartments can assemble in a host cell and
392 that protein cargo can be included (Choudhary *et al.*, 2012; Lee *et al.*, 2012). The key trick is to
393 ensure that the shell proteins contain suitable pores for transport of RuBP and bicarbonate
394 into the microcompartment and 3-PGA out while trapping the released CO₂ effectively. An
395 alternative strategy is to explore the utility of scaffolding RuBisCO and CA so that CO₂ is
396 released in close proximity to RuBisCO, thus decreasing oxygenase activity (Singleton, Harmer,
397 Porter and Smirnov, unpublished results; <http://magic.psrg.org.uk>). This approach has
398 theoretical problems because the inherently slow catalytic rate of RuBisCO prevents effective
399 use of CO₂ before it diffuses away. Other synthetic biology approaches to improving
400 photosynthesis such as expression of carboxysome-like structures in chloroplasts may also
401 emerge in the near future.

402

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406

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Table 1. Evidence for metabolons and metabolic channeling in plants. The nature of the evidence is as follows: A, immunogold localisation; B, physical association (co-purification/pulldown/yeast 2-hybrid); C, co-localisation of fluorescently tagged proteins *in vivo* (e.g. FRET/BIFC); D, flux measurements.

Pathway	Evidence	References
Glycolysis	B, C, D	(Giege <i>et al.</i> , 2003; Graham <i>et al.</i> , 2007)
Calvin-Benson cycle	A, D	(Anderson <i>et al.</i> , 2006; Suss <i>et al.</i> , 1993; Szecowka <i>et al.</i> , 2013)
Phenylpropanoids	B, C, D	(Achnine <i>et al.</i> , 2004; Bassard <i>et al.</i> , 2012; Rasmussen and Dixon, 1999)
Flavonoids	B,C	(Crosby <i>et al.</i> , 2011)
Spermine/spermidine	B	(Panicot <i>et al.</i> , 2002)
Indole acetic acid	B	(Müller and Weiler, 2000)
Long chain alkanes	B, C	(Bernard <i>et al.</i> , 2012)
Sporopollenin	B, C	(Lallemand <i>et al.</i> , 2013)
Glyoxylate cycle	B	(Beeckmans <i>et al.</i> , 1994)
Dhurrin (cyanogenic glycoside)	B, C	(Nielsen <i>et al.</i> , 2008)