

REVIEW ARTICLE

DOI: 10.1038/s41467-018-04543-8

OPEN

The role of dynamic enzyme assemblies and substrate channelling in metabolic regulation

Lee J. Sweetlove¹ & Alisdair R. Fernie²

Transient physical association between enzymes appears to be a cardinal feature of metabolic systems, yet the purpose of this metabolic organisation remains enigmatic. It is generally assumed that substrate channelling occurs in these complexes. However, there is a lack of information concerning the mechanisms and extent of substrate channelling and confusion regarding the consequences of substrate channelling. In this review, we outline recent advances in the structural characterisation of enzyme assemblies and integrate this with new insights from reaction-diffusion modelling and synthetic biology to clarify the mechanistic and functional significance of the phenomenon.

Metabolism is central to cellular function, growth and survival. Hundreds of chemical reactions occur simultaneously to process energy sources and to provide precursor molecules for cell construction and chemical defence. The majority of these reactions are accelerated and regulated by enzymes that not only allow metabolism to occur on an appropriate timescale for life but also allow the outputs of the metabolic system to be specified and controlled. Moreover, enzymes allow feedback loops and other regulatory mechanisms to operate so that the system can maintain homeostasis under variable conditions. Enzymes are highly effective catalysts and biochemical reactions are massively accelerated. Nevertheless, few enzymes have attained catalytic perfection and catalysis remains orders of magnitude slower than the diffusion of the substrates and products. Hence, the long-standing view of metabolic systems is that they are ‘well mixed’ with homogeneously distributed enzymes and freely diffusing metabolites.

This view of metabolism is the main basis for modelling of enzyme kinetics¹ and indeed holds true for purified enzymes in the test tube. However, over the past 40 years, evidence has steadily accumulated to suggest that this view is not an entirely accurate description of metabolism *in vivo*. Repeated observations have been made which suggest that enzymes are not always homogeneously distributed in the cell but rather can come together in time and space, due to protein–protein interactions between them. The enzymes in such assemblies usually constitute recognised metabolic pathways and their formation and disassembly is dynamically responsive to biochemical conditions and stimuli. Moreover, metabolites may be channelled between sequential enzymes: *i.e.* the product of one enzyme is transferred to the next enzyme in the pathway without equilibrating with the bulk aqueous phase of the cell. In this case, the assemblies are known as metabolons, a term coined by Paul Srere some 40 years ago² who provided the first detailed study of the phenomenon while working on the mitochondrial tricarboxylic acid (TCA) cycle. Since these pioneering studies, assemblies of consecutive enzymes have been observed in a wide variety of pathways, including glycolysis, oxidative

¹Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK. ²Max-Planck-Institut für Molekulare Pflanzenphysiologie, Potsdam-Golm 14476, Germany. Correspondence and requests for materials should be addressed to L.J.S. (email: lee.sweetlove@plants.ox.ac.uk) or to A.R.F. (email: fernie@mpimp-golm.mpg.de)

Table 1 A representative list of dynamic enzyme assemblies, the species in which, and the method(s) by which, they were detected

Pathway	Method of detection	Species	Reference
Oxidative pentose phosphate pathway	Isotope dilution studies	Pea, soybean, yeast	83
Glycolysis	Cell biology, affinity purification mass spectrometry, isotope dilution studies	<i>Arabidopsis thaliana</i> , human cell lines, human heart, potato, yeast, <i>Synechocystis</i> sp.	57,84–86
TCA cycle	Cell biology, affinity purification mass spectrometry, isotope dilution studies, site-directed mutagenesis, kinetic modelling, protein crystallisation	<i>Arabidopsis</i> , <i>Bacillus subtilis</i> , potato, <i>S. cerevisiae</i> , <i>E. coli</i> , rat	40–42,45,67,82,87–91
CoA channelling through FA synthesis	Substrate pool size quantification and enzyme kinetics	Pea and spinach	92
FA β -oxidation cycle	Protein crystallisation	<i>E. coli</i>	93
Polyketide pathway	Enzyme kinetic analysis	<i>Saccharopolyspora erythraea</i>	94
Purinosome	Cell biology, proteomics, mutant analysis, comparison of enzyme assembly with the rate of flux through the pathway	Human cell lines	49–51,95
Mitochondrial ETC complexes	Enzyme assays in the presence/absence of electron donors and inhibitors and genetically modified supercomplexes	Mouse fibroblasts	96
Photosynthesis			
(i) Light-harvesting complex	Spectroscopic analysis of isolated protein complexes and genetically modified complexes	<i>Chlamydomonas reinhardtii</i>	97
(ii) Calvin–Benson cycle	Isotope dilution, proteomics	<i>Synechocystis</i> sp.	98
Cyanogenic glucoside biosynthesis	Isotope dilution, enzyme kinetic analysis, co-purification studies	Sorghum	99,100
Phenylpropanoid biosynthesis	Channelling of labelled intermediates, cell fractionation experiments, co-immunolocalisation	Plants including <i>Arabidopsis</i> , buckwheat, petunia, maize and snapdragon	101,102
Polyamine biosynthesis	Yeast two hybrid, co-immunoprecipitations, cell biology	<i>Arabidopsis</i> , <i>Leishmania donovani</i>	103,104
Branched chain amino acid metabolism	Protein association studies, site directed mutational analysis	Isolated proteins from human and rat	105
Alkaloid	Cell biology	<i>Catharanthus roseus</i>	106
Tetrapyrrole biosynthesis	Crystallisation and structure determination	<i>Synechocystis</i> sp.	107
Lignin	Genetic manipulation, mathematical modelling	<i>Medicago truncatula</i>	68,108
Cell wall degradation	Cell biology, proteomics, crystallisation studies	<i>Arabidopsis</i> , rice, anaerobic bacteria and fungi	109
Proline catabolism	Kinetic considerations, effects of mutating one of the enzymes on the other, modelling based on available crystal structures	<i>Thermus thermophilus</i>	110
RNA degradation	Cell biology, proteomics, crystallisation studies	<i>E. coli</i> , <i>Pseudomonas syringae</i> , <i>Rhodobacter capsulatus</i> , <i>Streptomyces coelicolor</i> , <i>Mycobacterium tuberculosis/bovis</i>	111

phosphorylation, fatty acid, amino acid, polyketide, polyamine and polypeptide biosynthesis^{3,4}. Enzyme assemblies have also been reported in photosynthesis and natural product metabolons in plants^{5,6} (see also Table 1 for details). Moreover, broad-scale screens using human cells have suggested the presence of up to 130,000 binary protein–protein interactions at any one time^{7,8}, underlining the commonality of such interactions. It is now apparent that enzyme assemblies are a cardinal feature of metabolism across all domains of life and occur both in central metabolism and in the specialised metabolism of plants and fungi^{6,9} (Table 1).

In this review, we consider the nature and functional consequences of organising enzymes into such assemblies, by drawing on recent experimental and theoretical advances. We will discuss two different types of assembly: (i) those built from specific pairwise interactions between sequential enzymes with complementary structural features; (ii) larger clusters formed from multiple copies of all enzymes of a pathway. These two types of assembly have different implications for substrate channelling, pathway flux and regulation of flux at metabolic network branch points and these will be considered in detail. We will illustrate each type of assembly by considering naturally occurring examples (focussing mainly on the TCA cycle and purine biosynthesis,

where recent advances in the structure and function of the assemblies have been made) as well as examples from synthetic biology. Finally, we will consider the relevance of enzyme assemblies for metabolic engineering as well as discussing the crucial experimental evidence that will be required to fully understand the biochemical importance of this enigmatic phenomenon. Owing to space constraints, we cannot cover every reported dynamic superstructure nor can we discuss every feature of such assemblies but the interested reader is referred to several excellent recent reviews that cover these aspects in more detail^{10–13}.

The enigmatic and misunderstood phenomenon of substrate channelling

The most common explanation for the dynamic formation of enzymes into physical assemblies is that it promotes substrate channelling—that is, the facilitated transfer of the metabolite product from one enzyme to the next enzyme in the pathway without that metabolite equilibrating with the bulk aqueous solvent. This can be particularly beneficial if the channelled metabolite is labile or toxic, which is quite commonly the case in plant and fungal secondary metabolism⁶. It also has obvious

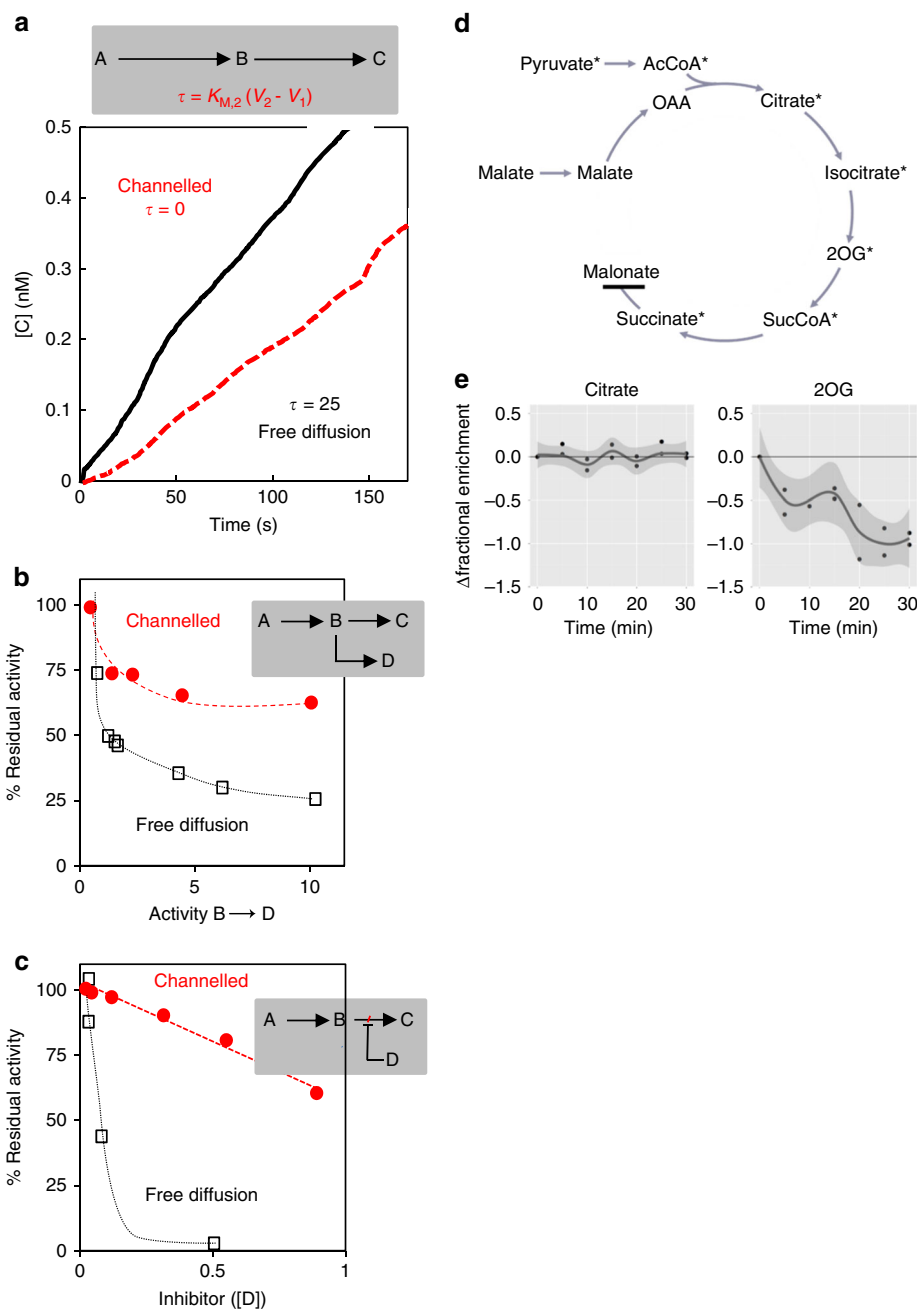


Fig. 1 Methods of identifying substrate channelling. **a** Reaction scheme and depiction of transient time (t) analysis based on data from a channelled bifunctional thymidylate synthase-dihydrofolate reductase (TS-DHFR) and a freely diffusing monofunctional TS and DHFR (data from ref. 64). **b** Comparison of residual activity of a channelled or freely diffusing enzyme pair in the presence of a competing enzyme, for example, the malate dehydrogenase and citrate synthase couple in the presence or absence of aspartate aminotransferase, which competes for the metabolic intermediate oxaloacetate (data from ref. 82). **c** Comparison of residual activity of a channelled or freely diffusing enzyme pair in the presence of an inhibitor of the second enzyme, for example, the inhibition of the TS-DHFR cascade by the inhibition of DHFR by pyruvate (data from ref. 64). **d** Schematic representation of the isotope dilution experiment to assess the channelling of citrate and 2OG. ^{13}C -labelled pyruvate was fed to isolated potato mitochondria and the label accumulation in succinate was monitored. The TCA cycle was inhibited by malonate to avoid the complication of multiple turns of the cycle. Non-labelled citrate and 2OG were added into the medium following the fractional enrichment in succinate reaching steady state. Asterisks show the expected fate of labelled carbon following the metabolism of pyruvate under the experimental conditions. **e** The result of isotope dilution experiments for citrate and 2OG. The time course plots show the fractional ^{13}C enrichment in succinate following the addition of unlabelled citrate or 2OG at 0 min. The line is the smoothed conditional mean with the shadow representing a 95% confidence interval. The metabolite is considered not to be channelled when the confidence interval line falls below 0. Panels **a-c** has been adapted with permission from ref. 10. Panels **d, e** has been reproduced with permission from ref. 41

implications for regulation of flux at branch points in the metabolic network—a channelled metabolite is not available for use by a competing enzyme at a branch point. This feature of substrate channelling will be discussed in more detail later.

But first we wish to address a major, and widely held, misconception about substrate channelling: which is that substrate channelling makes the metabolic pathway more efficient or kinetically superior¹⁴. Instinctively, this feels correct. Surely

enzymes must work faster if substrates are passed between them without diffusing away into the bulk aqueous phase of the cell? However, for this to be true, diffusion would have to be a limiting factor in non-channelled reactions, and in most cases, it is not. Diffusion of metabolites is extremely fast in relation to the rate of catalysis by enzymes, even when the crowded and viscous environment of the cell is taken into account. Various calculations have been made that demonstrate this. For example, the calculated diffusion rate for collisions between enzymes and their metabolites is estimated to be 1000–10,000 times faster than the typical K_{cat}/K_M value for enzymes of central metabolism¹². Alternatively, it has been estimated the time taken for metabolites to diffuse between enzymes is one-to-three orders of magnitude faster than the average reaction turnover time for enzymes of central metabolism^{12,15}. Similarly, a diffusion model was used to demonstrate that the concentration of product diffusing from an enzyme active site remains constant at distances of a few micrometres away¹⁰. Given that an enzyme concentration of 1 nM corresponds to an inter-enzyme distance of approximately 1 μm between enzymes, it is evident that diffusion is amply fast enough to evenly distribute metabolites between enzymes and does not limit their catalytic rate. In other words, it is reasonable to conclude that, diffusion is not the limiting factor for the rate of a reaction and hence metabolite channelling will not increase the rate of the reaction at steady state.

There are two exceptions in which metabolite channelling may accelerate the steady-state reaction rate. First, there are a small number of enzymes that are deemed to be catalytically perfect and whose K_{cat}/K_M ratio is sufficiently high that the reaction does become limited by diffusion of the reactants¹⁶. A notable example in central metabolism is the glycolytic enzyme, triose phosphate isomerase. The second exception is if enzyme concentration is very low, meaning that the average distances between enzymes can increase to the point that the reaction is partially diffusion limited. This is considered to be a more relevant scenario for specialised metabolism of plants and fungi¹².

Why do synthetic enzyme assemblies go faster?

Over the past few years, there have been a growing number of experiments in which two or more enzymes of a metabolic pathway have been co-localised onto synthetic scaffold biomolecules^{17,18}. These experiments are often predicated on the notion that enzyme proximity will lead to substrate channelling and that this will accelerate reaction rate. However, as we have already discussed, substrate channelling will not lead to a faster steady-state reaction rate than achieved by the freely diffusing enzymes unless the freely diffusing enzymes are at very low concentrations or are catalytically perfect enzymes. Given that these conditions do not apply to most of the published enzyme-scaffolding studies, then how to we reconcile the fact that almost all of these studies report several-fold enhancements of reaction rate for the scaffolded enzymes? Rate enhancements have been observed for scaffolds made from protein, lipid or DNA/RNA^{19,20} and for substrates with widely different physicochemical properties²¹.

This apparent contradiction has prompted a reassessment of the nature of the rate enhancement in scaffolded reactions, using both modelling²² and experimental approaches^{23,24}. This has provided a new perspective on the behaviour of co-localised enzymes that is relevant to our understanding of the function of *in vivo* enzyme assemblies. First, in enzyme-scaffolding experiments, reaction rate is typically monitored after initiating the reaction. Although the reaction is usually followed for tens of minutes thereafter, many reactions take hours to reach steady state *in vitro*. For example, a kinetic model of the commonly used glucose oxidase–horseradish peroxidase pair shows that, for experimentally relevant enzyme

concentrations, the reaction takes >5 h to reach steady state²². In other words, much of the published data for scaffolded enzymes may be following the reaction during its ‘lag-phase’ prior to steady state. Therefore, the observed rate enhancement could be due to substrate channelling due to its effect of shortening the lag time (see Fig. 1a for a more detailed explanation). Nevertheless, there remain conceptual doubts as to whether channelling is likely in these systems^{11,22}.

Moreover, it is also possible that alterations in enzyme kinetics could explain the enhancement of initial rate. Unfortunately, there is a lack of experimental assessment of this issue in the scaffolding literature. For a more detailed analysis of the effect of altered enzyme kinetics on reaction rates over time, we refer the reader to an excellent recent review¹¹. In fact, there is evidence that immobilised enzymes can have superior kinetics²⁵, although the underlying explanation for this remains unclear. It may be that immobilising the enzyme to a scaffold changes the local biophysical conditions resulting in a microenvironment that is closer to the intracellular environment. In other words, the enhanced kinetics of scaffolded enzymes are closer to *in vivo* kinetics than those measured *in vitro* for dilute solutions of purified enzymes. If true, this would have far-reaching consequences for kinetic modelling of metabolic networks. In a recent study of glucose oxidase and horseradish peroxidase enclosed in DNA ‘nanocages’, it was found that, when each enzyme was enclosed in a separate cage, the activity of the reaction was four-fold higher than for freely diffusing enzymes²⁶. Importantly, the enclosed enzymes had a higher reaction than enzymes bound to a ‘half-cage’ that was open to the bulk solution, supporting a microenvironment-based explanation for altered enzyme kinetics. One intriguing suggestion as to the nature of this microenvironment effect is that the regularly spaced phosphates of the DNA scaffold decreases the local pH, which increase the reaction rate of this enzyme pair²³. Indeed, tuning of the local pH environment around each enzyme in a reaction cascade has recently been shown to be an effective way of increasing reaction rate²⁷. Another possible mechanism of altering of the kinetics is that enzyme assemblies may prevent compounds from exerting an inhibitory effect on the enzyme by restricting them from access to the active site of the enzyme⁶. It is thus likely that a range of biophysical/microenvironment effects could be at play to explain the increased initial reaction rate observed across multiple scaffold types and structures and different enzyme pairs.

Substrate channelling mechanism

Insights from the TCA cycle. Substrate channelling has benefits other than increasing reaction flux and there is abundant evidence that it does operate in naturally occurring enzyme assemblies. Perhaps the best-studied example of specific physical interactions between sequential enzymes associated with substrate channelling is the TCA cycle. Collectively, the body of work on the TCA cycle metabolon by Srere, Sumegi and their co-workers has been absolutely fundamental in setting our contemporary views concerning enzyme interactions and substrate channelling^{2,28}. The general advantages of such channels have been mentioned above, namely, regulating metabolic flux at branch points as well as in specific cases being involved in the sequestration of labile and/or toxic intermediates. In addition, enzyme assemblies have been documented to influence allosteric effects (see, for example, ref. 6). There is a wealth of evidence from biochemical experiments for interactions between TCA cycle enzymes^{2,28} and between TCA cycle enzymes and inner mitochondrial membrane proteins^{29–32} or members of the mitochondrial carrier family^{33,34}. In conjunction, there is a convincing body of evidence from enzyme kinetic and isotope labelling

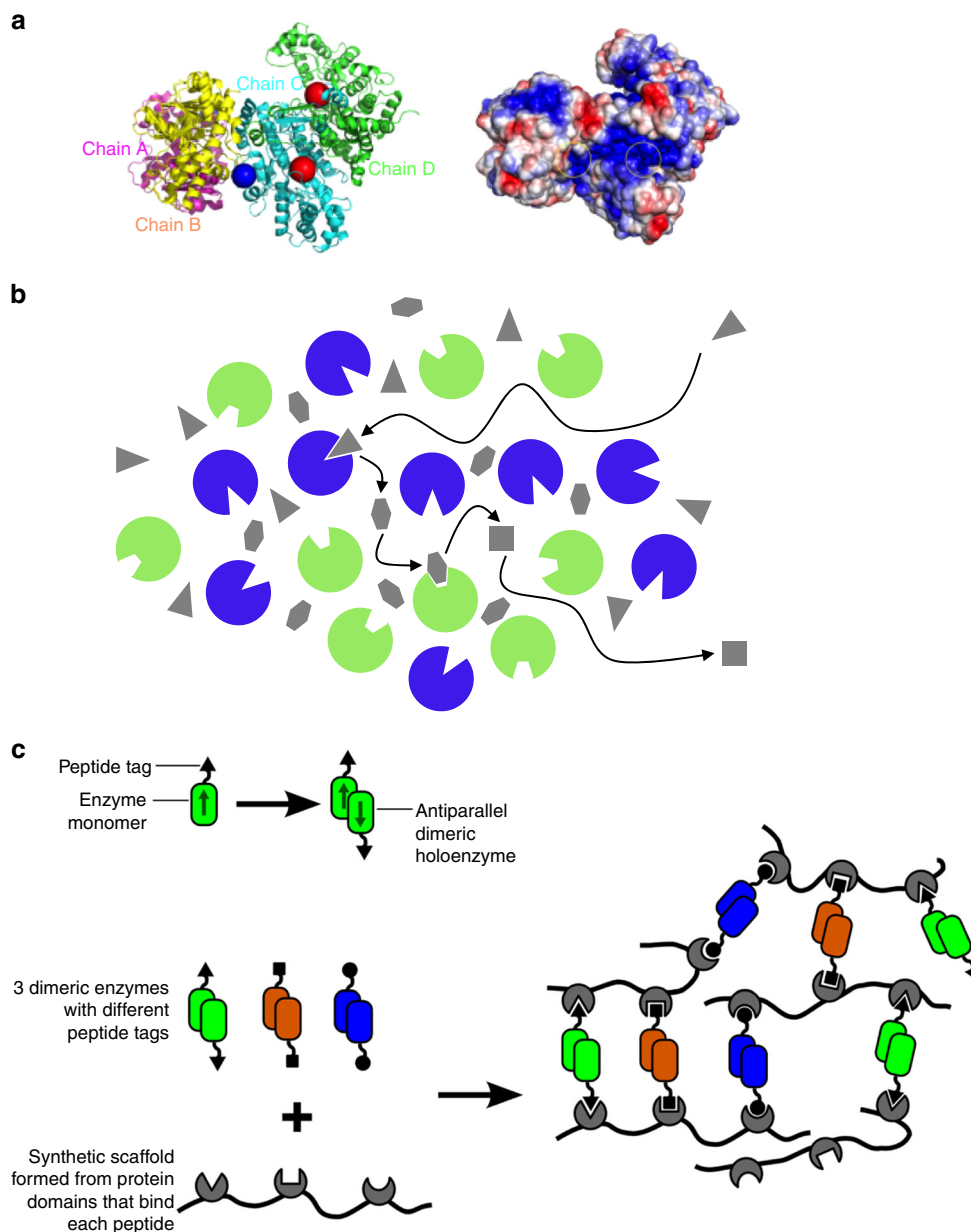


Fig. 2 Mechanisms of substrate channelling in dynamic enzyme assemblies. **a** Direct channelling by electrostatic retention of the channelled metabolite on the surface of the enzyme complex. A structural model of the bovine malate dehydrogenase (MDH)-citrate synthase (CS) complex is shown. On the left, the polypeptides are illustrated as ribbon diagrams, with the MDH dimer shown in magenta and yellow and the CS dimer show in green and cyan. The blue circle shows where OAA molecules were initially placed in a Brownian dynamics simulation. Red circles show the active sites in the CS dimer. On the right, the surface structure of the complex is shown, with red and blue colours representing negative and positive electrostatic potential, respectively. Neutral regions are shown in white. Yellow circles indicate the positions of the adjacent MDH and CS active sites. **b** Probabilistic channelling within a large cluster of enzymes. Two enzymes are shown as green and blue circles. Metabolites are shown as grey polygons, with each shape representing a different metabolite. The arrows indicate the path taken by metabolites in a sequential conversion event by two enzymes. **c** Mechanism of enzyme cluster formation in synthetic scaffold-enzyme assemblies. When oligomeric enzymes are docked onto synthetic protein scaffolds via peptide tags, then interaction with more than one scaffold molecule are possible leading to the formation of a large aggregation of scaffolded enzyme complexes

studies that strongly suggests metabolites are channelled between the enzymes of the cycle^{31,35–39}. These classical studies have been bolstered by more recent work using modern techniques in cell biology and proteomics. For instance, binary interactions have been systematically assessed between *Bacillus subtilis* TCA cycle enzymes using a combination of bacterial two-hybrid analysis and affinity purification–mass spectrometry. Interactions were identified between six consecutive enzymes of the TCA cycle (linking fumarate to succinyl CoA), as well as interactions of these enzymes with phosphoenolpyruvate carboxykinase and glutamate

synthase⁴⁰. In plants, a comprehensive range of techniques including affinity purification–mass spectrometry, split-luciferase and yeast two-hybrid assays have recently been utilised to demonstrate protein–protein interactions in the TCA cycle⁴¹.

Until recently, however, the mechanistic basis by which these enzyme–enzyme interactions facilitates substrate channelling has not been clear. Substrate channelling requires the metabolite product of one enzyme to be passed as substrate to the next enzyme in the pathway while being prevented from diffusing into the bulk solvent. In stable multi-enzyme complexes, the reaction

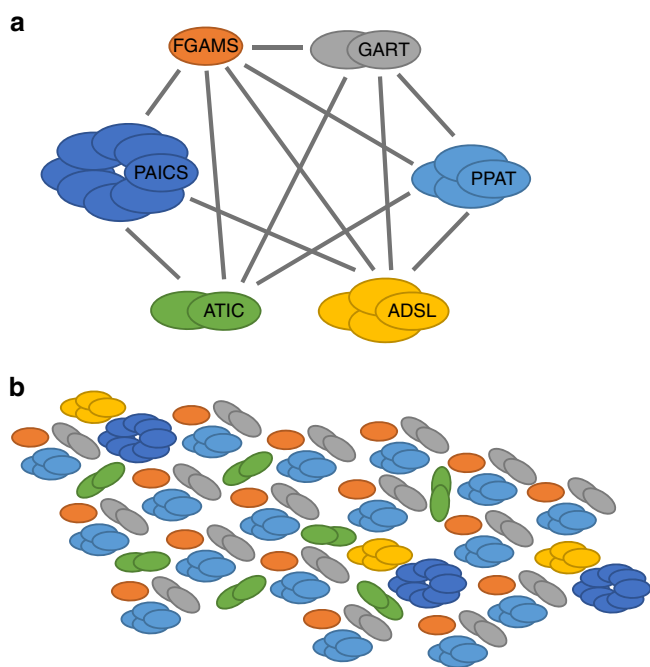


Fig. 3 The purinosome—a naturally occurring enzyme cluster? **a**

Protein–protein interactions within the purinosome. Each holoenzyme of the purine biosynthesis pathway is shown, with the number of subunits reflecting the dominant oligomeric state of each. The grey lines indicate experimentally demonstrated pairwise protein–protein interactions. PPAT phosphoribosylpyrophosphate amidotransferase, ADSL adenylosuccinate lyase, ATIC 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/5'-inosinemonophosphate cyclohydrolase, FGAMS phosphoribosyl formylglycinamide synthase, GART phosphoribosylglycinamide synthetase/phosphoribosylglycinamide formyltransferase/phosphoribosylaminoimidazole synthetase, PAICS phosphoribosyl aminoimidazole succinocarboxamide synthetase. **b** Potential organisation of the purine biosynthesis enzymes within the purinosome enzyme cluster. The first three enzymes of the pathway, PPAT, GART and FGAMS, form a core complex that interacts with an ADSL–PAICS pair and with ATIC. In this way, multiple copies of the core complex are assembled via interactions with ADSL–PAICS and ATIC

intermediates are entirely enclosed in internal tunnels or chambers within the protein complex, allowing directed diffusion between active sites or the use of swing arms to physically mobilise intermediates or cofactors^{10,15}. However, there is no evidence of such structures forming due to dynamic pairwise interactions of TCA cycle enzymes.

A recent structural analysis of the malate dehydrogenase–citrate synthase–aconitase complex has provided insight into the potential mechanism by which substrate channelling could occur (Fig. 2a)⁴². In this study, *in vivo* crosslinking and mass spectrometry were used to establish a low-resolution structure of the complex. Crosslinking suggested that all eight enzymes of the cycle physically interact, but only the interactions between malate dehydrogenase and citrate synthase were sufficiently strong to allow their recovery in non-crosslinked samples. Using distance constraints derived from the crosslinking process, models of the malate dehydrogenase–citrate synthase–aconitase complex were proposed. The average distances between active sites were 35 Å between malate dehydrogenase and citrate synthase and 50 Å between aconitase and citrate synthase. Analysis of the modelled superstructure revealed that polar residues are considerably over-represented on its surface while less-polar residues tend to be

even more buried than is usually the case. Moreover, these positively charged residues are arrayed within a groove that connects the malate dehydrogenase and citrate synthase active sites. Hence, complementary structural and charge features of the interacting enzymes provide a mechanism to promote efficient transfer of the negatively charged oxaloacetate intermediate between adjacent active sites. Electrostatic retention of the intermediate on the enzyme surface would not only reduce equilibration of the intermediate with the bulk, but theoretical models suggest that it would promote efficient transfer between the two enzymes⁴³, provided that the electrostatic transfer groove is not too long⁴⁴.

In a follow-up study, site-directed mutagenesis was performed on the highly conserved arginine residues located in the positively charged groove^{42,45}. Mutation of one of these residues in the citrate synthase polypeptide to an uncharged residue (R65A) did not affect catalytic activity of the enzyme but reduced the probability of channelling from 0.99 to 0.023. Another intriguing feature which became apparent in these studies was that recombinant versions of malate dehydrogenase and citrate synthase self-assemble *in vitro*. From this work, it can be concluded that these enzymes have co-evolved complementary structural features that allow them to interact in such a way as to align surface features that may promote substrate channelling.

Enzyme clusters or microdomains. One surprising feature of the more recent systematic studies of protein–protein interactions in the TCA cycle was the extensive interactions between non-sequential pathway enzymes in addition to the expected interactions between sequential enzymes⁴¹. It is conceivable that many of these interactions, which occur largely between regulatory subunits and catalytic subunits of different proteins, act as nucleation points that aid in the formation of the specific interactions between the sequential enzymes⁴¹. However, it is also possible that these additional interactions give rise to a different type of structure to the classical pairwise interaction: an enzyme cluster or enzyme microdomain. This is a larger assembly of multiple copies of each enzyme of a pathway as illustrated in Fig. 2b. This may or may not contain specific pairwise interactions within it and may or may not be ordered. The enzyme cluster leads to substrate channelling by a different mechanism and also has implications for reaction rate.

The concept of an enzyme cluster arose in the synthetic biology literature in which three enzymes of the mevalonic acid pathway were docked onto a protein scaffold by virtue of a peptide–protein interaction⁴⁶. Recombinant enzymes were created with the relevant peptide tag at their C-terminus. However, because each of the holoenzymes is a homodimer with the two subunits in anti-parallel orientation, the scaffolded enzymes can interact with a second scaffold. Given the flexible nature of the scaffolds used, each enzyme does not necessarily interact with the same two scaffold molecules and it is therefore possible that a large cluster of physically connected enzyme–scaffold complexes can form⁴⁷ (Fig. 2c).

In this cluster, substrate channelling can still occur but no longer requires specific pairwise interaction between sequential enzymes. Instead, the effect is probabilistic: when the metabolite product of an enzyme reaction diffuses away from the active site of one enzyme in a cluster, it has a much greater probability of encountering the next enzyme in the pathway than diffusing into the bulk aqueous solvent of the cell. This has been demonstrated in a reaction–diffusion model of a scaffolded enzyme system⁴⁸. The model also suggested that the overall reaction rate would be enhanced. The explanation for the enhanced rate is simple: by bringing enzymes together into clusters, rather than having them

distributed through the cell, the local concentration of enzymes is increased. This leads to a kinetic benefit for enzyme systems that are reaching substrate saturation. The model suggests that flux would increase by 6-fold for a 2-step pathway and over 100-fold for a 3-step pathway compared to freely diffusing enzymes within a cell⁴⁸. Note that this is in contrast to the situation for enzyme assemblies formed solely from pairwise interactions where direct substrate channelling does not increase the steady-state reaction rate.

The purinosome—a naturally occurring enzyme cluster?

Recent work on purine nucleotide synthesis suggests that enzyme clusters are not only confined to synthetic enzyme assemblies but can also occur in the natural world. Purine nucleotides are a key component of DNA and RNA and also act as energy carriers and cofactors required to promote cell survival and proliferation. Remarkably, under cellular circumstances that lead to a high demand for purines, the enzymes of de novo purine biosynthesis cluster together into multi-enzyme complexes that have been dubbed purinosomes⁴⁹. Confocal microscopy of HeLa cells revealed co-location of all six enzymes of the pathway in clusters of 0.2–0.9 μm in diameter under conditions where purine biosynthesis demand is high⁵⁰. Proteomics has subsequently been used to define the nature of protein–protein interactions within the cluster demonstrating that a core complex which assembles in a step-wise fashion includes the first three enzymes in the pathway (Fig. 3a). Subsequent study of mutants of other enzymes in the pathway revealed a decreased purinosome formation suggesting that these proteins likely affect complex stability⁵¹. Further analysis of the assembly of the purinosome has implicated the Hsp70/Hsp90 chaperone machinery⁵² as well as casein kinase⁵³, microtubules⁵³ and G-protein coupled receptors⁵⁴ in the process. Schematic representations of the purinosome complex typically show only a single copy of each holoenzyme (Fig. 3a). However, the purinosome complex as observed in vivo has an average diameter of 0.56 μm , which is sufficient room to contain many copies of the individual enzymes. If we assume that the 6 holoenzymes of the purinosome are spheres with an average diameter of 10 nm and are packed into a cubic volume at a packing density of 64% (the maximum packing density for irregular packing of spheres), then they would fit into a cube with edges of 17 nm in length, suggesting that 33 copies of each enzyme could be present (see Supplementary Note for details of this calculation). This is a minimum estimate given that the structure of the protein complexes is likely to allow closer packing density and protein stoichiometry is not necessarily equal, i.e. it is conceivable that many more copies of the three core-complex proteins could be present. Another way of looking at this is to consider that cells typically contain around 1 million protein molecules per μm^3 ⁵⁵. Hence the volume occupied by the purinosome (0.0092 μm^3 for a sphere of diameter 0.56 μm) has room for 9200 individual proteins. Taking these calculations into account and what is known about the protein–protein interactions between the purine biosynthesis enzymes (Fig. 3a), we suggest that hundreds of copies of the three core-complex enzymes are present in the purinosome, brought together by interactions with the remaining enzymes of the pathway (Fig. 3b).

Further work is required to establish the exact architecture and size of the purinosome complex but it seems likely that the purinosome is a naturally occurring example of an enzyme cluster in which reaction rate is enhanced by increased enzyme concentration and substrate channelling is probabilistic rather than direct. Consistent with this, the formation of the purinosome in vivo is coincident with a 50% increase in measured flux through de novo purine biosynthesis⁵⁶. The fact that the

purinosome is formed when purine synthesis demand is high and is associated with increased flux suggests that purinosome formation is likely to be a means of controlling pathway flux.

Dynamic enzyme assemblies control flux at network branch points

Whether one is considering enzyme assemblies formed by pairwise interactions or larger clusters of enzymes, an important feature of these complexes is that they effectively change the structure of the metabolic network due to substrate channelling. Specifically, they bypass branch points in the metabolic network: the channelled intermediate is prevented from being utilised in competing branch-point reactions.

Hence, the dynamic formation of enzyme assemblies is potentially an important regulatory feature allowing the relative flux in different branches of metabolic pathways to be controlled. This is critical when there is a necessity to rapidly increase the flux through one of the branches at the expense of the other. For example, substrate channelling between enzymes of glycolysis when located on the surface of mitochondria has been proposed to be a regulatory mechanism to ensure that sufficient pyruvate is supplied to the mitochondria when respiratory demand is increased, rather than allowing intermediates of glycolysis to be used for competing pathways, such as amino acid biosynthesis⁵⁷.

Recently, the concept of substrate channelling as a branch-point regulatory mechanism has been directly experimentally tested by examining the effect of clustering of enzymes at a branch point between pyrimidine and arginine synthesis⁴⁸. The branch point occurs at the metabolite, carbamoyl phosphate (CMP). Substrate channelling of CMP between the pyrimidine pathway enzymes CMP synthase (CMPS) and aspartate–CMP–transferase (ACMPT) would direct flux towards pyrimidine synthesis and prevent the arginine pathway enzyme ornithine–CMP–transferase competing for CMP. This flux change can be experimentally detected in bacteria as arginine pseudo-auxotrophy (i.e. growth is stimulated by the addition of arginine to the medium) and an insensitivity to the addition of uracil, a pyrimidine precursor. To test this, a genetic fusion of CMPS and ACMPT was expressed in a CMPS/ACMPT knockout strain of *Escherichia coli*. This did not result in arginine pseudo-auxotrophy, suggesting that the two enzymes lack structural compatibility/surface features to create channelling directly. However, when the fusion protein was more strongly overexpressed, protein clusters were visible under phase-contrast microscopy, assumed to be μm -scale assemblies of the CMPS–ACMPT fusion. In this situation, arginine pseudo-auxotrophy was observed, with the degree of growth stimulation by exogenous arginine correlated with the extent of protein cluster formation. This mechanism of flux control at branch points is now beginning to be incorporated into metabolic engineering experiments. For example, it was shown that flux from pyruvate to various fermentation end products in *E. coli* could be controlled by creating complexes of pyruvate kinase and the pyruvate-consuming enzymes of different fermentation branches⁵⁸.

In the context of the purinosome and TCA cycle, there are known branch points in the local metabolic network that potentially could be controlled by this mechanism. In purine biosynthesis, the intermediate S-aminimidazole ribonucleotide is a substrate for a reaction in the pathway of thiamine biosynthesis⁵⁹. And the TCA cycle intermediate, oxaloacetate, is also used for aspartate biosynthesis⁶⁰. Hence, in scenarios such as purine depletion where it is necessary to rapidly increase the flux of purine biosynthesis, the formation of the purinosome achieves this by two mechanisms: by increasing local enzyme concentration and by preventing competition for intermediates by branch-point pathways.

Characterising the functional properties of dynamic enzyme assemblies

Enzyme assemblies have been characterised at the functional level, focussing mainly on substrate channelling, and at the structural level. It is clear that, if we are to better understand how these enzyme assemblies work, then the two facets need to be brought together. In the case of the TCA cycle, structural studies are bringing new insights into the potential mechanism of substrate channelling, but as of yet, the effect of mutation of structural features on substrate channelling have not been directly tested and so proof of the proposed mechanism is missing. In the case of the purinosome, there have been no direct tests of the effects of the complex on enzyme kinetics or substrate channelling. And more work is required to define the exact structure of the assembly and in particular whether it contains multiple copies of enzymes as we have suggested here. Another highly interesting aspect of the formation of dynamic enzyme assemblies that is receiving increasing attention is the influence of ionic and organic solute environments on their formation^{45,61}. Intriguingly, high concentrations of compatible solutes not only promote enzyme folding and stability but also potentially alter the effective enzyme concentration that may affect oligomerisation and kinetics^{62,63}, which may extend to associations between enzymes. The recent demonstration of a substrate driven chemostatic assembly of the enzyme cascade linking the first four activities of glycolysis (ref. 61), as well as the malate dehydrogenase–citrate synthase–aconitase complex assembly of the TCA cycle⁴⁵, highlight the influence of small molecule metabolites on setting gradients aiding in dynamic enzyme assemblies. Although in a well-mixed cellular environment, it is unclear how relevant this gradient-driven assembly will be.

Going beyond the structural–functional level, the most important unanswered question of all is the role of enzyme assembly in the context of cellular biochemistry. We have proposed control of flux at metabolic branch points as a key role, but other advantages of such enzyme assemblies such as protection of labile reactants are also conceivable. Indeed, the latter has been suggested in the context of the purinosome where the product of the first enzyme of the pathway has a short solution half-life⁴⁹. None of the proposed functional benefits of enzyme assemblies have been directly tested. To do this, one would ideally exploit the molecular mechanism that controls assembly and disassembly of the complex. Manipulation/mutation of this mechanism would allow the consequences of prevention of the enzyme assembly formation to be investigated and the biochemical role of the assembly to be established.

The other key question that needs to be addressed is how prevalent such enzyme assemblies are in nature. Protein–protein interaction studies have suggested that they are remarkably widespread and perhaps even the rule rather than the exception. But few of these interactions have been functionally assessed and this is largely due to the technical limitations and difficulties associated with classical methods of assessing substrate channelling. The methods available to biochemists for assessing substrate channelling are summarised in Fig. 1. For example, features of reaction kinetics such as the lag time (also known as the transient time, τ) can be used as an indirect method of demonstrating substrate channelling. Following initiation of a two-step reaction, there is a lag before the reaction reaches a steady state in which the intermediate metabolite concentration reaches a constant value dictated by the kinetics of the producing and consuming enzyme and the size of the reaction container. If channelling occurs, the reaction container volume is effectively reduced and the reaction therefore reaches steady state much more rapidly. In a perfectly channelled system, the lag time approaches zero (Fig. 1a). However, as already discussed, changes in kinetic properties and concentrations of the enzymes also affect the lag time and in many

cases appropriate controls to account for this are missing¹¹. An alternative kinetic method is to perturb the reaction from the bulk environment, as exemplified in Fig. 1b, c, which show the perturbation by a competing enzyme or the presence of an inhibitory molecule within the bulk environment, respectively. The first approach has been used to assess channelling mediated by a malate dehydrogenase–citrate synthase couple in the presence or absence of aspartate amino transferase³⁹, while the second was applied to the bifunctional thymidylate synthase–dihydrofolate reductase in the presence and absence of trimethoprim and pyrimethamine⁶⁴. Ultimately, these methods are generally limited to *in vitro* studies and are complicated by the fact that one is looking for the absence of an effect as evidence of substrate channelling³⁹.

A more useful approach involves isotopically labelled substrates. In the ‘isotope dilution’ method, an isotope-labelled precursor to the pathway of interest is supplied to cells or tissues and the dilution of label in downstream pathway products following addition of an unlabelled pathway intermediate to the bulk solvent is monitored. Substrate channelling reduces or prevents equilibration of the bulk-solvent unlabelled intermediate with the equivalent channelled intermediate and so reduces or prevents isotope dilution⁶⁵. The approach has two main limitations: first, many metabolic intermediates are not effectively taken up from the exogenous medium. This can be dealt with by permeabilisation treatments or expression of broad specificity transporters, as performed in studies of channelling in bacterial glycolysis⁶⁶. The second problem is that the labelling patterns following addition of the unlabelled intermediate can be difficult to interpret, depending on the extent to which the enzymes under consideration are saturated with substrate, for example. Labelling patterns can be further confounded by cyclical pathways where multiple rounds of the cycle can lead to loss of label or excessive randomisation. The latter issue was addressed in a recent study of substrate channelling in the plant TCA cycle by using inhibitors to linearise the cycle. Isolated potato tuber mitochondria were supplied with ¹³C-labelled pyruvate or glutamate until accumulation of ¹³C in TCA cycle intermediates reached isotopic steady state (Fig. 1d, e). Then unlabelled intermediates of the TCA cycle were added and the dilution effect on labelling was monitored over time. For the pyruvate feeding experiments, the TCA cycle was linearised by inhibiting succinate dehydrogenase with malonate and, for the glutamate experiments, by inhibiting aconitase with fluoroacetate⁴¹. These experiments revealed dilution in 2-oxoglutarate, succinate and malate but none in fumarate or citrate, indicating channelling of these two intermediates between the respective enzyme pairs.

It should be obvious from these discussions that new methods are needed that would allow substrate channelling to be assessed *in vivo*, at a wider scale and with greater throughput. A new approach that has potential to achieve this has recently been described and is based upon the well-established methodologies of ¹³C-metabolic flux analysis (MFA). In MFA, a ¹³C-labelled metabolic precursor that is readily taken up is supplied and the pattern of redistributed label at isotopic steady state is analysed. Labelling patterns are then simulated in a metabolic network model and fluxes in the model iteratively varied until a good fit between the predicted and measured labelling patterns is achieved. Because substrate channelling changes the structure of the metabolic network where it bypasses branch points, the approach can be used to determine whether substrate channelling is required to account for the labelling patterns and, if so, can provide a quantitative estimate of the substrate channelled flux *in vivo*. This method was applied to heterotrophic *Arabidopsis* cell cultures and revealed substrate channelling of fumarate to malate but no channelled flux from 2OG or succinate to citrate⁶⁷ in the plant TCA cycle.

A related modelling-based approach for quantitative assessment of channelling at the metabolic network scale is to construct an enzyme kinetic model and to assess whether the introduction of specific channelled pathways improves the ability of the model to predict observed data sets. This approach was used to assess channelling between two branch points in lignin biosynthesis in plants⁶⁸. Depending on the operation of the channelled pathways, 19 different configurations of the lignin biosynthesis network could be formulated. These were each tested in a large set of Generalised Mass Action models that accounted for saturable enzyme kinetics and aspects of regulatory crosstalk between the two channelled branches. The models were benchmarked against a data set of lignin content and monomer composition (guaiacyl (G), and syringyl (S) monomers) in transgenic alfalfa lines with reduced expression of different enzymes in the lignin pathway. The modelling suggested that channelling is necessary but not sufficient to explain the results of the transgenic plants and that specific crosstalk between the two channelled pathways is also required. Additionally, the modelling revealed that operation of the G-lignin channel was more important for an accurate description of the data than the S-lignin channel.

Summary and outlook

The study of dynamically formed enzyme assemblies has a long history but it is only in recent years that structural features of these assemblies have begun to be elucidated. Coupled with a growing body of experimental work exploring the properties of synthetic enzyme assemblies, we are now able to provide a more

integrated picture of the function of these transient enzyme complexes. Using the purinosome and the TCA cycle as examples, we have described two types of enzyme assembly, one formed from large clusters of multiple copies of enzymes and the other formed from single complexes of paired enzymes with complementary surface features that serve to direct diffusion of the channelled intermediate. We have argued that the role of these enzyme assemblies is regulatory. In the case of large enzyme clusters, the increase in local enzyme concentration is expected to increase pathway flux. And in both types of complex, effective channelling of intermediates (either directly between paired enzymes or probabilistically in large enzyme clusters) bypasses branch points in the local network, allowing flux through the channelled branch to be increased at the expense of the competing branch.

A number of other pertinent questions also remain to be addressed including the precise roles of sub-metabolons and the role of scaffolding proteins, described to be present in both the purinosome and TCA cycle assemblies, remains unclear. As does the role of membranes that are associated with both of these assemblies and many of the others listed in Table 1. Moreover, studies to define the influence of post-translational modifications such as reversible changes in structure or phosphorylation will likely be highly informative. Once the residues important for interaction and those important for protein modifications have been defined, the advent of genome-editing techniques should render the testing of their functional significance relatively facile.

A more complete understanding of the role of enzyme assemblies will also be crucial for engineering of metabolic

Box 1. | An evolutionary perspective of enzyme complexes

Here we review literature concerning the evolutionary histories of dynamic assemblies of enzymes and stable multi-enzyme complexes. More research of an evolutionary bent has been carried out on the latter—many of which are highly conserved throughout evolution. For example, a pioneering study suggested that the α -ketoacid dehydrogenase complexes appeared early in evolution, being found in aerobic members of the archaea, bacteria and eukaryotes⁷¹. Another multi-enzyme complex that has been subject to particular scrutiny is the fatty acid synthase complex (FAS)—of which two strikingly different types have evolved in eukaryotes: the metazoan and fungal FAS⁷². The metazoan FAS is a 540-kDa homodimer that shares a common architecture with bacterial polyketide synthases⁷³, while the fungal FAS forms a 2.6-MDa assembly comprising 48 functional domains and, alongside the homologous CMN-FAS recently described in *Corynebacterium*, *Mycobacterium* and *Nocardia*⁷⁴, constitute some of the most complex biochemical protein machineries known⁷⁵. A recent study identified bacterial non-canonical fatty acid synthases and *trans*-acting polyketide enoyl reductases as potential ancestors of the scaffolding regions with striking conservation of insertions to scaffolding elements despite minimal sequence identity⁷². In contrast, plants and many bacteria harbour only monofunctional FASs⁷⁶. Similarly, the penta-functional Arom complex of shikimate biosynthesis appears likely to have evolved via gene fusion with the enzymes of most other species being monofunctional⁹. A final multi-enzyme complex that merits discussion is that of tryptophan synthase⁷⁷. In their study, Leopoldseder et al. suggest that it is plausible to assume that sophisticated multi-enzyme complexes evolve via stepwise association of protein subunits (see Supplementary Figure 3)⁷⁸.

The evolution of metabolons is much less studied. However, as indicated in Table 1, these display a similar range of conservation throughout evolution as the stable multi-enzyme complexes. In particular, metabolons within the TCA cycle and glycolysis are highly conserved. Others, such as the urea/polyamine metabolon, are found, at least in part, in several species. Other metabolons, especially those involved in plant and fungal specialised metabolism, are far more restricted in their species range. However, despite the widespread occurrence of metabolons (dynamic enzyme assemblies) and stable multi-enzyme complexes, there is no reason to suspect that the evolution of the former is an obligatory step towards the evolution of the latter. Indeed, it is likely that the two types of enzyme assemblies have evolved to play quite distinct functions from one another.

The presence of protein aggregates has been argued to be under negative selection. However, protein aggregation may also be unavoidable (for example, owing to the limited solvency capacity of the cell in relation to the protein concentration) and therefore functional organisation around such aggregates may represent an evolutionary compromise⁷⁹. Given that many oligomeric proteins may adopt multiple structural conformations, there may be unique opportunities for mutation to lead to the formation of new protein–protein contact points. While in many cases new aggregations will be deleterious, some will be neutral and even ultimately beneficial to the cell⁷⁹, in which case one would anticipate their stabilisation. Recent studies of protein interaction data in the context of the evolutionary properties of the interaction network have left several unanswered questions⁸⁰. Most pertinent of these being (i) to what extent do protein interactions act as constraints during the evolution of the protein sequence; (ii) what role do transient or obligate interactions play in these constraints; (iii) are mutations in the binding site of an interacting protein correlated in those in the binding site of its partner. Intriguingly, a study aimed at addressing these questions observed that residues at the interfaces of obligate complexes evolved at a relatively slower rate allowing them to co-evolve with their interacting partners. In contrast, where the protein–protein interactions are transient, the rate of residue substitution is much higher⁸¹. These observations are thus in line with our suggestion that multi-enzyme complexes and metabolons evolve separately and result in distinctive repurposing of the ancestral enzymes, from one another. We propose that the key functional discriminator is the requirement for access of competing enzymes to the channelled metabolite so that metabolic branch points can operate. This would require disassembly of the enzyme complex that supports substrate channelling and would provide a selective pressure against the evolution of a stable multi-enzyme complex for that set of reactions. In other words, stable multi-enzyme complexes can only evolve where there are no other cellular reactions that requires access to the channelled metabolite.

networks and for the successful introduction of synthetic metabolic pathways into host organisms. Indeed it is possible that proteins within synthetic scaffolds are turned over at a decreased rate than the native enzymes. Failure to account for the presence of enzyme assemblies and substrate channelling will lead to flawed designs for engineered pathways and problematic interactions between synthetic pathways and host metabolism. For example, it has been suggested that substrate channelling in glycolysis prevents bacteria such as *E. coli* from utilising C5 sugars as fermentation substrates¹², a major target for the biofuel industry allowing the use of plant lignocellulosic feedstocks. The argument is that the degradation products of C5 sugars are prevented from entering metabolism due to the channelling of glycolysis. Hence, it may be necessary to disrupt this endogenous channel in order to engineer bacteria that can efficiently ferment C5 sugars.

A final consideration is the potential link between substrate channelling and regulation of metabolism at the gene-expression level via metabolite signalling. Metabolite signalling is the greatest challenge for the metabolic engineer: current mathematical models of the combinatorial and non-linear gene-regulatory responses to changes in metabolite concentration are insufficient to allow accurate predictions of the outcome of genetic intervention in the metabolic network⁶⁹. A recent breakthrough study of transcriptional regulation of metabolism in *E. coli* revealed that most of the metabolite feedback to the genetic regulatory system occurred via just three metabolites—cyclic AMP, fructose 1-phosphate and fructose 1,6-bisphosphate⁷⁰. Given that substrate channelling effectively renders channelled metabolites invisible to metabolite signalling systems, this remarkable discovery may point towards a greater extent of substrate channelling in central metabolism than has hitherto been suspected, with the channelling effectively directing metabolic fluxes between a small number of non-channelled metabolite nodes upon which regulation is focussed (Box 1).

Received: 14 November 2017 Accepted: 8 May 2018

Published online: 30 May 2018

References

- Voit, E. O. The best models of metabolism. *Wiley Interdiscip. Rev. Syst. Biol. Med* **9**, e1391 (2017).
- Srere, P. A. The metabolon. *Trends Biochem. Sci.* **10**, 109–110 (1985).
- Costa, A., Hood, I. V. & Berger, J. M. Mechanisms for initiating cellular DNA replication. *Annu. Rev. Biochem.* **82**, 25–54 (2013).
- de la Cruz, J., Karbstein, K. & Woolford, J. L. Functions of ribosomal proteins in assembly of eukaryotic ribosomes in vivo. *Annu. Rev. Biochem.* **84**, 93–129 (2015).
- Shen, J. R. The structure of photosystem II and the mechanism of water oxidation in photosynthesis. *Annu. Rev. Plant Biol.* **66**, 23–48 (2015).
- Jorgensen, K. et al. Metabolon formation and metabolic channeling in the biosynthesis of plant natural products. *Curr. Opin. Plant. Biol.* **8**, 280–291 (2005).
- Bonetta, L. Interactome under construction. *Nature* **468**, 851–854 (2010).
- Havugimana, P. C. et al. A census of human soluble protein complexes. *Cell* **150**, 1068–1081 (2012).
- Vandenhommergh, J., Moore, J. D., Charles, I. G. & Hawkins, A. R. Overproduction in *Escherichia coli* of the dehydroquinase synthase domain of the *Aspergillus nidulans* pentafunctional arom protein. *Biochem. J.* **284**, 861–867 (1992).
- Wheeldon, I. et al. Substrate channelling as an approach to cascade reactions. *Nat. Chem.* **8**, 299–309 (2016).
A comprehensive review of substrate channelling, the mechanisms behind it and the analytical methods to assess it as well as an appreciation of the harnessing of this phenomenon in synthetic biology approaches.
- Zhang, Y. F. & Hess, H. Toward rational design of high-efficiency enzyme cascades. *ACS Catal.* **7**, 6018–6027 (2017).
- Abernathy, M. H., He, L. & Tang, Y. J. Channeling in native microbial pathways: implications and challenges for metabolic engineering. *Biotechnol. Adv.* **35**, 805–814 (2017).
- Schmitt, D. L. & An, S. Spatial organization of metabolic enzyme complexes in cells. *Biochemistry* **56**, 3184–3196 (2017).
- Ovadi, J. & Saks, V. On the origin of intracellular compartmentation and organized metabolic systems. *Mol. Cell. Biochem.* **256**, 5–12 (2004).
- Sweetlove, L. J. & Fernie, A. R. The spatial organization of metabolism within the plant cell. *Annu. Rev. Plant Biol.* **64**, 723–746 (2013).
- Petterson, G. Evolutionary optimization of the catalytic efficiency of enzymes. *Eur. J. Biochem.* **206**, 289–295 (1992).
- Agapakis, C. M., Boyle, P. M. & Silver, P. A. Natural strategies for the spatial optimization of metabolism in synthetic biology. *Nat. Chem. Biol.* **8**, 527–535 (2012).
- Pröschel, M., Detsch, R., Boccaccini, A. R. & Sonnwald, U. Engineering of metabolic pathways by artificial enzyme channels. *Front. Bioeng. Biotechnol.* **3**, 168 (2015).
- Myhrvold, C., Polka, J. K. & Silver, P. A. Synthetic lipid-containing scaffolds enhance production by colocalizing enzymes. *ACS Synth. Biol.* **5**, 1396–1403 (2016).
- Delebecque, C. J., Lindner, A. B., Silver, P. A. & Aldaye, F. A. Organization of intracellular reactions with rationally designed RNA assemblies. *Science* **333**, 470–474 (2011).
- Sachdeva, G., Garg, A., Godding, D., Way, J. C. & Silver, P. A. In vivo colocalization of enzymes on RNA scaffolds increases metabolic production in a geometrically dependent manner. *Nucleic Acids Res.* **42**, 9493–9503 (2014).
- Idan, O. & Hess, H. Origins of activity enhancement in enzyme cascades on scaffolds. *ACS Nano* **7**, 8658–8665 (2013).
- Zhang, Y. F., Tsvitkov, S. & Hess, H. Proximity does not contribute to activity enhancement in the glucose oxidase-horseradish peroxidase cascade. *Nat. Commun.* **7**, 13982 (2016). **Using the glucose oxidase and horseradish peroxidase reaction cascade revealed no difference in the kinetics of the cascade reaction when the enzymes are free in solution, or when they are conjugated to each other and when a competing enzyme is present thus challenging the notion of a proximity effect promoting activity within enzyme cascades.**
- Poshyvailo, L., von Lieres, E. & Kondrat, S. Does metabolite channeling accelerate enzyme-catalyzed cascade reactions? *PLoS ONE* **12**, e0172673 (2017).
- Johnson, B. J., Algar, W. R., Malanoski, A. P., Ancona, M. G. & Medintz, I. L. Understanding enzymatic acceleration at nanoparticle interfaces: approaches and challenges. *Nano Today* **9**, 102–131 (2014).
- Zhao, Z. et al. Nanocaged enzymes with enhanced catalytic activity and increased stability against protease digestion. *Nat. Commun.* **7**, 10619 (2016).
- Zhang, Y. F., Wang, Q. & Hess, H. Increasing enzyme cascade throughput by ph-engineering the microenvironment of individual enzymes. *ACS Catal.* **7**, 2047–2051 (2017).
- Srere, P. A. Complexes of sequential metabolic enzymes. *Annu. Rev. Biochem.* **56**, 89–124 (1987).
- Sumegi, B. & Srere, P. A. Binding of the enzymes of fatty-acid β -oxidation and some selected enzymes to the inner mitochondrial membrane. *Biophys. J.* **45**, A250 (1984).
- Sumegi, B. & Srere, P. A. Complex-I binds several mitochondrial NAD-coupled dehydrogenases. *J. Biol. Chem.* **259**, 5040–5045 (1984).
- Kispal, G., Sumegi, B. & Alkonyi, I. Isolation and characterization of 3-hydroxyacyl coenzyme-A dehydrogenase-binding protein from pig-heart inner mitochondrial-membrane. *J. Biol. Chem.* **261**, 14209–14213 (1986).
- Dsouza, S. F. & Srere, P. A. Cross-linking of mitochondrial matrix proteins in situ. *Biochim. Biophys. Acta* **724**, 40–51 (1983).
- Grigorenko, E., Small, W., Persson, L. & Srere, P. Citrate synthase 1 interacts with the citrate transporter of yeast mitochondria. *J. Mol. Recognit.* **3**, 215–219 (1990).
- Beeckmans, S. & Kanarek, L. Demonstration of physical interactions between consecutive enzymes of the citric-acid cycle and of the aspartate-malate shuttle—a study involving fumarase, malate-dehydrogenase, citrate synthase and aspartate-aminotransferase. *Eur. J. Biochem.* **117**, 527–535 (1981).
- Srere, P. A., Mattiasson, B. & Mosbach, K. Immobilized 3-enzyme system—model for microenvironmental compartmentation in mitochondria. *Proc. Natl. Acad. Sci. USA* **70**, 2534–2538 (1973).
- Sumegi, B., Sherry, A. D. & Malloy, C. R. Channeling of TCA cycle intermediates in cultured *Saccharomyces cerevisiae*. *Biochemistry* **29**, 9106–9110 (1990).
- Porpaczy, Z. & Sumegi, B. Interaction between the alpha-ketoglutarate dehydrogenase complex and succinate thiokinase in the Krebs cycle. *Acta Biochim. Biophys. Hung.* **19**, 6 (1984).
- Morgunov, I. & Srere, P. A. Interaction between citrate synthase and malate dehydrogenase—substrate channeling of oxaloacetate. *J. Biol. Chem.* **273**, 29540–29544 (1998).

39. Spivey, H. O. & Ovadi, J. Substrate channeling. *Methods* **19**, 306–321 (1999). **A detailed and beautifully argued critique of the methods for assessing substrate channelling, the advantages and disadvantages they present and the quality of conclusions that can be drawn from them.**
40. Meyer, F. M. et al. Physical interactions between tricarboxylic acid cycle enzymes in *Bacillus subtilis*: evidence for a metabolon. *Metab. Eng.* **13**, 18–27 (2011).
41. Zhang, Y. J. et al. Protein-protein interactions and metabolite channelling in the plant tricarboxylic acid cycle. *Nat. Commun.* **8**, 15212 (2017). **A combination of cell biological, proteomic and flux profiling approaches are combined to demonstrate that in plants as in microbes and mammals the canonical metabolon of the tricarboxylic acid cycle is operational.**
42. Wu, F. & Minteer, S. Krebs cycle metabolon: structural evidence of substrate channeling revealed by cross-linking and mass spectrometry. *Angew. Chem. Int. Ed.* **54**, 1851–1854 (2015). **Chemical crosslinking and structural modelling of the Krebs cycle metabolon demonstrates electrostatic channelling to occur upon protein-protein association providing the first structural evidence of substrate channelling within this pathway.**
43. Eun, C., Kekenus-Huskey, P. M., Metzger, V. T. & McCammon, J. A. A model study of sequential enzyme reactions and electrostatic channeling. *J. Chem. Phys.* **140**, 105101 (2014).
44. Huang, Y. M. M., Huber, G. A., Wang, N., Minteer, S. D. & McCammon, J. A. Brownian dynamic study of an enzyme metabolon in the TCA cycle: substrate kinetics and channeling. *Protein Sci.* **27**, 463–471 (2018).
45. Wu, F., Pelster, L. N. & Minteer, S. D. Krebs cycle metabolon formation: metabolite concentration gradient enhanced compartmentation of sequential enzymes. *Chem. Commun.* **51**, 1244–1247 (2015).
46. Dueber, J. E. et al. Synthetic protein scaffolds provide modular control over metabolic flux. *Nat. Biotechnol.* **27**, 753–759 (2009). **This paper demonstrates the advantages for metabolic engineering of scaffolding proteins together in order to enhance the production of high-value compounds.**
47. Lee, H., DeLoache, W. C. & Dueber, J. E. Spatial organization of enzymes for metabolic engineering. *Metab. Eng.* **14**, 242–251 (2012).
48. Castellana, M. et al. Enzyme clustering accelerates processing of intermediates through metabolic channeling. *Nat. Biotechnol.* **32**, 1011–1019 (2014).
49. Pedley, A. M. & Benkovic, S. J. A new view into the regulation of purine metabolism: the purinosome. *Trends Biochem. Sci.* **42**, 141–154 (2017).
50. An, S., Kumar, R., Sheets, E. D. & Benkovic, S. J. Reversible compartmentalization of de novo purine biosynthetic complexes in living cells. *Science* **320**, 103–106 (2008).
51. Baresova, V. et al. Mutations of ATIC and ADSL affect purinosome assembly in cultured skin fibroblasts from patients with AICA-ribosiduria and ADSL deficiency. *Hum. Mol. Genet.* **21**, 1534–1543 (2012).
52. French, J. B. et al. Hsp70/Hsp90 chaperone machinery is involved in the assembly of the purinosome. *Proc. Natl. Acad. Sci. USA* **110**, 2528–2533 (2013).
53. An, S., Deng, Y., Tomsho, J. W., Kyoung, M. & Benkovic, S. J. Microtubule-assisted mechanism for functional metabolic macromolecular complex formation. *Proc. Natl. Acad. Sci. USA* **107**, 12872–12876 (2010).
54. Verrier, F. et al. GPCRs regulate the assembly of a multienzyme complex for purine biosynthesis. *Nat. Chem. Biol.* **7**, 909–915 (2011).
55. Milo, R., Jorgensen, P., Moran, U., Weber, G. & Springer, M. BioNumbers—the database of key numbers in molecular and cell biology. *Nucleic Acids Res.* **38**, D750–D753 (2010).
56. Zhao, H. et al. Quantitative analysis of purine nucleotides indicates that purinosomes increase de novo purine biosynthesis. *J. Biol. Chem.* **290**, 6705–6713 (2015).
57. Graham, J. W. A. et al. Glycolytic enzymes associate dynamically with mitochondria in response to respiratory demand and support substrate channeling. *Plant Cell* **19**, 3723–3738 (2007).
58. Kim, S., Bae, S. J. & Hahn, J. S. Redirection of pyruvate flux toward desired metabolic pathways through substrate channeling between pyruvate kinase and pyruvate-converting enzymes in *Saccharomyces cerevisiae*. *Sci. Rep.* **6**, 24145 (2016).
59. Kim, S., Bae, S. J. & Hahn, J. S. Thiamin (vitamin B1) biosynthesis and regulation: a rich source of antimicrobial drug targets? *Int. J. Biol. Sci.* **7**, 41–50 (2011).
60. Sullivan, L. B. et al. Supporting aspartate biosynthesis is an essential function of respiration in proliferating cells. *Cell* **162**, 552–563 (2015).
61. Zhao, X. et al. Substrate-driven chemotactic assembly in an enzyme cascade. *Nat. Chem.* **10**, 311–317 (2018). **This paper describes the application of microfluidic and fluorescent spectroscopy techniques to study the coordinated movement of the first four enzymes of the glycolytic cascade revealing, for the first time, that each enzyme independently follows its own specific substrate gradient.**
62. Podesta, F. E. & Andreo, C. S. Maize leaf phosphoenolpyruvate carboxylase—oligomeric state and activity in the presence of glycerol. *Plant Physiol.* **90**, 427–433 (1989).
63. Manetas, Y., Petropoulou, Y. & Karabourniotis, G. Compatible solutes and their effects on phosphoenolpyruvate carboxylase of C4-halophytes. *Plant Cell Environ.* **9**, 145–151, x (1986).
64. Trujillo, M., Donald, R. G. K., Roos, D. S., Greene, P. J. & Santi, D. V. Heterologous expression and characterization of the bifunctional dihydrofolate reductase-thymidylate synthase enzyme of *Toxoplasma gondii*. *Biochemistry* **35**, 6366–6374 (1996).
65. Moller, B. L. & Conn, E. E. The biosynthesis of cyanogenic glucosides in higher plants—channeling of intermediates in dhurrin biosynthesis by a microsomal system from *Sorghum bicolor* (Linn) Moench. *J. Biol. Chem.* **255**, 3049–3056 (1980).
66. Shearer, G., Lee, J. C., Koo, J. & Kohl, D. H. Quantitative estimation of channeling from early glycolytic intermediates to CO₂ in intact *Escherichia coli*. *FEBS J.* **272**, 3260–3269 (2005).
67. Williams, T. C. R., Sweetlove, L. J. & Ratcliffe, R. G. Capturing metabolite channeling in metabolic flux phenotypes. *Plant Physiol.* **157**, 981–984 (2011).
68. Lee, Y., Escamilla-Trevino, L., Dixon, R. A. & Voit, E. O. Functional analysis of metabolic channeling and regulation in lignin biosynthesis: a computational approach. *PLoS Comput. Biol.* **8**, e1002769 (2012).
69. Nielsen, J. & Keasling, J. D. Engineering cellular metabolism. *Cell* **164**, 1185–1197 (2016).
70. Kochanowski, K. et al. Few regulatory metabolites coordinate expression of central metabolic genes in *Escherichia coli*. *Mol. Syst. Biol.* **13**, 903 (2017).
71. Mooney, B. P., Miernyk, J. A. & Randall, D. D. The complex fate of alpha-ketoacids. *Annu. Rev. Plant Biol.* **53**, 357–375 (2002).
72. Bukhari, H. S. T., Jakob, R. P. & Maier, T. Evolutionary origins of the multienzyme architecture of giant fungal fatty acid synthase. *Structure* **22**, 1775–1785 (2014).
73. Maier, T., Jenni, S. & Ban, N. Architecture of mammalian fatty acid synthase at 4.5 angstrom resolution. *Science* **311**, 1258–1262 (2006).
74. Gago, G., Diacovich, L., Arabolaza, A., Tsai, S.-C. & Gramajo, H. Fatty acid biosynthesis in actinomycetes. *FEMS Microbiol. Rev.* **35**, 475–497 (2011).
75. Grininger, M. Perspectives on the evolution, assembly and conformational dynamics of fatty acid synthase type I (FAS I) systems. *Curr. Opin. Struct. Biol.* **25**, 49–56 (2014). **A fascinating review of the evolution and assembly of the largest known enzyme assembly—fatty acids synthases type I that provides a useful framework for the study of other multi-enzyme complexes and dynamic enzyme assemblies alike.**
76. Campbell, J. W. & Cronan, J. E. Bacterial fatty acid biosynthesis: targets for antibacterial drug discovery. *Annu. Rev. Microbiol.* **55**, 305–332 (2001).
77. Leopoldseder, S., Hettwer, S. & Sterner, R. Evolution of multi-enzyme complexes: the case of tryptophan synthase. *Biochemistry* **45**, 14111–14119 (2006).
78. Dandekar, T., Snel, B., Huynen, M. & Bork, P. Conservation of gene order: a fingerprint of proteins that physically interact. *Trends Biochem. Sci.* **23**, 324–328 (1998).
79. O’Connell, J. D., Zhao, A., Ellington, A. D. & Marcotte, E. M. Dynamic reorganization of metabolic enzymes into intracellular bodies. *Annu. Rev. Cell Dev. Biol.* **28**, 89–111 (2012).
80. Fraser, H. B., Hirsh, A. E., Steinmetz, L. M., Scharfe, C. & Feldman, M. W. Evolutionary rate in the protein interaction network. *Science* **296**, 750–752 (2002).
81. Mintseris, J. & Weng, Z. P. Structure, function, and evolution of transient and obligate protein-protein interactions. *Proc. Natl. Acad. Sci. USA* **102**, 10930–10935 (2005).
82. Lindblad, C. et al. Preparation and kinetic characterization of a fusion protein of yeast mitochondrial citrate synthase and malate-dehydrogenase. *Biochemistry* **33**, 11692–11698 (1994).
83. Debnam, P. M., Shearer, G., Blackwood, L. & Kohl, D. H. Evidence for channeling of intermediates in the oxidative pentose phosphate pathway by soybean and pea nodule extracts, yeast extracts, and purified yeast enzymes. *Eur. J. Biochem.* **246**, 283–290 (1997).
84. Taylor, S. W. et al. Characterization of the human heart mitochondrial proteome. *Nat. Biotechnol.* **21**, 281–286 (2003).
85. Giege, P. et al. Enzymes of glycolysis are functionally associated with the mitochondrion in *Arabidopsis* cells. *Plant Cell* **15**, 2140–2151 (2003).
86. Brandina, I. et al. Enolase takes part in a macromolecular complex associated to mitochondria in yeast. *Biochim. Biophys. Acta* **1757**, 1217–1228 (2006).
87. Velot, C., Mixon, M. B., Teige, M. & Srere, P. A. Model of a quinary structure between Krebs TCA cycle enzymes: a model for the metabolon. *Biochemistry* **36**, 14271–14276 (1997).
88. Sumegi, B., Sherry, A. D., Malloy, C. R. & Srere, P. A. Evidence for orientation-conserved transfer in the TCA cycle in *Saccharomyces cerevisiae*—C-13 NMR studies. *Biochemistry* **32**, 12725–12729 (1993).

89. Sumegi, B., Sherry, A. D., Malloy, C. R., Evans, C. & Srere, P. A. Is there tight channeling in the tricarboxylic-acid cycle metabolon. *Biochem. Soc. Trans.* **19**, 1002–1005 (1991).
90. Bulutoglu, B., Garcia, K. E., Wu, F., Minter, S. D. & Banta, S. Direct evidence for metabolon formation and substrate channeling in recombinant TCA cycle enzymes. *ACS Chem. Biol.* **11**, 2847–2853 (2016).
91. Niklas, J., Sandig, V. & Heinzle, E. Metabolite channeling and compartmentation in the human cell line AGE1.HN determined by C-13 labeling experiments and C-13 metabolic flux analysis. *J. Biosci. Bioeng.* **112**, 616–623 (2011).
92. Roughan, P. G. Stromal concentrations of coenzyme A and its esters are insufficient to account for rates of chloroplast fatty acid synthesis: evidence for substrate channelling within the chloroplast fatty acid synthase. *Biochem. J.* **327**, 267–273 (1997).
93. Ishikawa, M., Tsuchiya, D., Oyama, T., Tsunaka, Y. & Morikawa, K. Structural basis for channelling mechanism of a fatty acid beta-oxidation multienzyme complex. *EMBO J.* **23**, 2745–2754 (2004).
94. Wu, N., Tsuji, S. Y., Cane, D. E. & Khosla, C. Assessing the balance between protein-protein interactions and enzyme-substrate interactions in the channeling of intermediates between polyketide synthase modules. *J. Am. Chem. Soc.* **123**, 6465–6474 (2001).
95. French, J. B. et al. Spatial colocalization and functional link of purinosomes with mitochondria. *Science* **351**, 733–737 (2016).
Using super-resolution microscopy the authors demonstrated that purinosomes colocalized with mitochondria and, moreover, that the number of purinosome-containing cells responded to dysregulation of mitochondrial function and metabolism.
96. Lapuente-Brun, E. et al. Supercomplex assembly determines electron flux in the mitochondrial electron transport chain. *Science* **340**, 1567–1570 (2013).
97. Iwai, M., Yokono, M., Inada, N. & Minagawa, J. Live-cell imaging of photosystem II antenna dissociation during state transitions. *Proc. Natl. Acad. Sci. USA* **107**, 2337–2342 (2010).
98. Huege, J. et al. Modulation of the major paths of carbon in photorespiratory mutants of *Synechocystis*. *PLoS ONE* **6**, e016278 (2011).
99. Laursen, T. et al. Characterization of a dynamic metabolon producing the defense compound dhurrin in sorghum. *Science* **354**, 890–893 (2016).
100. Laursen, T., Moller, B. L. & Bassard, J. E. Plasticity of specialized metabolism as mediated by dynamic metabolons. *Trends Plant. Sci.* **20**, 20–32 (2015).
101. Diharce, J., Golebiowski, J., Fiorucci, S. & Antonczak, S. Fine-tuning of microsolvation and hydrogen bond interaction regulates substrate channelling in the course of flavonoid biosynthesis. *Phys. Chem. Chem. Phys.* **18**, 10337–10345 (2016).
102. Dastmalchi, M., Bernards, M. A. & Dhaubadel, S. Twin anchors of the soybean isoflavonoid metabolon: evidence for tethering of the complex to the endoplasmic reticulum by IFS and C4H. *Plant J.* **85**, 689–706 (2016).
103. Mishra, A. K., Agnihotri, P., Srivastava, V. K. & Pratap, J. V. Novel protein-protein interaction between spermidine synthase and S-adenosylmethionine decarboxylase from *Leishmania donovani*. *Biochem. Biophys. Res. Commun.* **456**, 637–642 (2015).
104. Panicot, M. et al. A polyamine metabolon involving aminopropyl transferase complexes in *Arabidopsis*. *Plant Cell* **14**, 2539–2551 (2002).
105. Islam, M. M. et al. A novel branched-chain amino acid metabolon—protein-protein interactions in a supramolecular complex. *J. Biol. Chem.* **282**, 11893–11903 (2007).
106. Stavrinides, A. et al. Unlocking the diversity of alkaloids in *Catharanthus roseus*: nuclear localization suggests metabolic channeling in secondary metabolism. *Chem. Biol.* **22**, 336–341 (2015).
107. Chen, X. M. et al. Crystal structure of the catalytic subunit of magnesium chelatase. *Nat. Plants* **1**, 15125 (2015).
108. Chen, F. et al. Multi-site genetic modulation of monolignol biosynthesis suggests new routes for formation of syringyl lignin and wall-bound ferulic acid in alfalfa (*Medicago sativa* L.). *Plant J.* **48**, 113–124 (2006).
109. Doi, R. H. & Kosugi, A. Cellulosomes: plant-cell-wall-degrading enzyme complexes. *Nat. Rev. Microbiol.* **2**, 541–551 (2004).
110. Sanyal, N., Arentson, B. W., Luo, M., Tanner, J. J. & Becker, D. F. First evidence for substrate channeling between proline catabolic enzymes a validation of domain fusion analysis for predicting protein-protein interactions. *J. Biol. Chem.* **290**, 2225–2234 (2015).
111. Marcaida, M. J., DePristo, M. A., Chandran, V., Carpousis, A. J. & Luisi, B. F. The RNA degradosome: life in the fast lane of adaptive molecular evolution. *Trends Biochem. Sci.* **31**, 359–365 (2006).

Acknowledgements

The authors would like to thank Ron Milo, Weizmann Institute of Science for discussions regarding the number of proteins that can fit inside a defined volume.

Author contributions

The concept, writing, and revision of this review was performed with equal contribution from L.J.S. and A.R.F.

Additional information

Supplementary Information accompanies this paper at <https://doi.org/10.1038/s41467-018-04543-8>.

Competing interests: The authors declare no competing interests.

Reprints and permission information is available online at <http://npg.nature.com/reprintsandpermissions/>

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2018