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Metabolons, Enzyme–Enzyme Assemblies that Mediate Substrate Channeling, and Their Roles in Plant Metabolism

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ABSTRACT

Metabolons are transient multi-protein complexes of sequential enzymes that mediate substrate channeling. They differ from multi-enzyme complexes in that they are dynamic, rather than permanent, and as such have considerably lower dissociation constants. Despite the fact that a huge number of metabolons have been suggested to exist in plants, most of these claims are erroneous as only a handful of these have been proven to channel metabolites. We believe that physical protein–protein interactions between consecutive enzymes of a pathway should rather be called enzyme–enzyme assemblies. In this review, we describe how metabolons are generally assembled by transient interactions and held together by both structural elements and non-covalent interactions. Experimental evidence for their existence comes from protein–protein interaction studies, which indicate that the enzymes physically interact, and direct substrate channeling measurements, which indicate that they functionally interact. Unfortunately, advances in cell biology and proteomics have far outstripped those in classical enzymology and flux measurements, rendering most reports reliant purely on interactome studies. Recent developments in co-fractionation mass spectrometry will likely further exacerbate this bias. Given this, only dynamic enzyme–enzyme assemblies in which both physical and functional interactions have been demonstrated should be termed metabolons. We discuss the level of evidence for the manifold plant pathways that have been postulated to contain metabolons and then list examples in both primary and secondary metabolism for which strong evidence has been provided to support these claims. In doing so, we pay particular attention to experimental and mathematical approaches to study metabolons as well as complexities that arise in attempting to follow them. Finally, we discuss perspectives for improving our understanding of these fascinating but enigmatic interactions.

Keywords: protein–protein interaction, metabolon, substrate channeling

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INTRODUCTION

In 1970, the concept of physical enzyme–enzyme complexes was initially suggested by A.M. Kuzin and subsequently adopted in 1972 by P.A. Srere for the enzymes of the citric acid cycle (Srere, 1972). The ''metabolon'' was first defined in 1985 by Paul Srere during a lecture in Debrecen, Hungary as ''a supramolecular complex of sequential metabolic enzymes and cellular structural elements'' (Srere, 1985). The metabolon was subsequently suggested to be a temporary structural–functional complex formed between sequential enzymes of a metabolic pathway and defined as metabolic channeling (or metabolite channeling or substrate channeling), whereby the intermediates are maintained within the metabolon and the interconversions are catalyzed by sequential enzymes. Srere also suggested that the enzyme supercomplex is assembled by transient, protein-surface, structure-dependent interactions that maintain substrate channeling, thereby regulating metabolic flux through the association and dissociation of the components of the metabolon (Figure 1). Thirty-five years later, we cannot come up with a better description. Indeed, all of this information still holds true and very few of Srere's seminal ideas have been disproved (Fernie et al., 2018; Sweetlove and Fernie, 2018).

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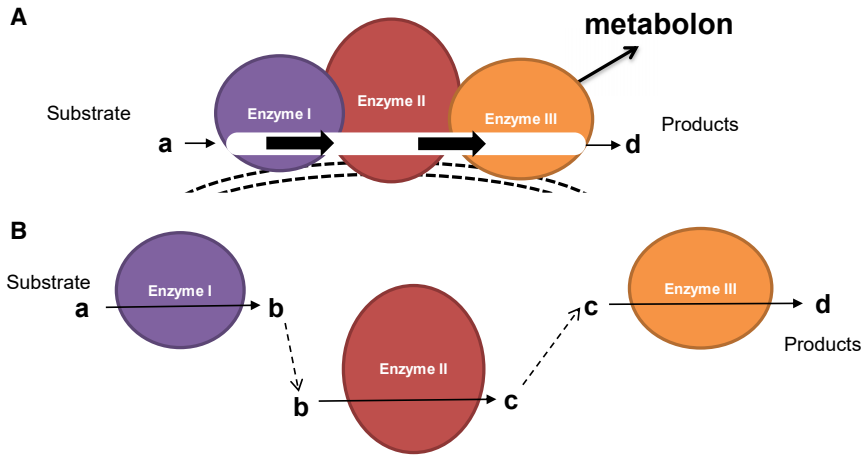

Figure 1. The Substrate Channeling Association.
Metabolic pathway in which product d is synthesized from substrate a via the reactions catalyzed by enzymes I, II, and III (arrows). b and c are pathway intermediates. **(A)** Association of the metabolon will enhance pathway reactions and is expected to upregulate the pathway. **(B)** Dissociation of the metabolon will downregulate the pathway.

Early studies have reported that substrate channeling is also linked with structural elements, such as integral membrane proteins and cytoskeletal proteins. Such interactions restrict the localization of enzymes, although common gene expression was initially supposed to play an important role in metabolon formation (Srere, 1985). In plants, there is weak circumstantial support for this, i.e., tricarboxylic acid (TCA) cycle enzymes are largely co-expressed (Cavalcanti et al., 2014), as will be discussed later; however, this is not necessarily the case. The first experimentally proven "metabolon" was the mitochondrial TCA cycle (Robinson and Srere, 1985). In this study, the authors reported that transient complexes can regulate metabolic pathway flux through dynamic associations and/or dissociations (Beeckmans et al., 1993; Velot et al., 1997; Fernie et al., 2018) (Figure 1). Subsequently, many protein complexes were proposed to mediate substrate channeling in various organisms, including the glycolytic pathway in mammals, yeast, and plants (Graham et al., 2007; Puchulu-Campanella et al., 2012; Araiza-Olivera et al., 2013), the TCA cycle in yeast and plants, branched-chain amino acid metabolism in human mitochondria (Islam et al., 2010), polyamine metabolism in plants (Panicot et al., 2002), and several different secondary metabolic pathways involving lignin, carotenoids, flavonoids, isoflavonoids, isoprenoids, alkaloids, camalexin, and cyanogenic glucoside synthesis in plants (Stavrinides et al., 2015; Laursen et al., 2016; Fujino et al., 2018; Gou et al., 2018; Camagna et al., 2019; Mucha et al., 2019). With a handful of exceptions, the evidence for their being metabolons is largely weak and, however attractive, the argument that their channeling substrates is definitive evidence that this is indeed the case is also weak. However, before we discuss this in detail, it is important to consider the potential advantage(s) of channeling.

During the formation of a substrate channel, the amount of water needed to hydrate enzymes is reduced; therefore, enzyme activity is increased. In this microenvironment within the substrate channel, various metabolic advantages have been proposed, such as increasing catalytic efficiency, enriching local substrates, protecting cells from cytotoxic intermediates, preventing the decomposition of unstable compounds, overcoming the thermodynamically unfavorable equilibrium, and avoiding competing pathways (Fernie et al., 2018). However, strictly speaking, substrate channeling must be observed to claim the existence of a metabolon according to the definition of Srere, and therefore not all enzyme–enzyme complexes are metabolons. To date, there is limited evidence—as a minimum, this must include proof of the formation of a transient protein complex and metabolite channeling—for the presence of metabolons. To our best knowledge, only the glycolytic pathway (Graham et al., 2007), TCA cycle (Zhang et al., 2017), early steps of phenylpropanoid biosynthesis (Achnine et al., 2004), and cyanogenic glucoside biosynthetic pathway (Laursen et al., 2016) meet these criteria in plants. As we have previously argued, mere demonstration of protein–protein interactions (PPIs) in dynamic enzyme assemblies is insufficient evidence for the existence of metabolons (Fernie et al., 2018; Sweetlove and Fernie, 2018).

Indeed, far greater proof must be obtained to substantiate the occurrence of substrate channeling. Methods that have been employed to date include (1) isotope dilution and enrichment, (2) a reaction scheme, followed by transient time analysis to compare residual activity between a channeled or freely diffusing enzyme pair, (3) a comparison of an enzyme pair, followed by challenge from a competing side reaction or cascade inhibitor, and (4) a model of NADH transfer (Wheeldon et al., 2016). None of these methods are facile, and the interpretation of their results is often complicated, as they mainly address if the pathway channeled or not? In most cases, the very nature of metabolons as transient enzyme assemblies means that at any one time, molecules of any enzyme species will simultaneously be both free and in a complex. Thus, substrate channels will likely only be identified via such methods in cases where a large proportion of the enzymes is bound. This, in itself, is not a significant problem because, for a metabolon to be physiologically relevant, it must account for a reasonable proportion of an enzyme species, at least locally. Otherwise, any benefit of such an association would be minimized by the fact that the majority of pathway flux is borne by the free enzymes. However, recent developments in co-fractionation mass spectrometry (MS), such as those described by McWhite and colleagues (McWhite et al., 2020; Zhang et al., 2020b), have revealed the protein–protein interactomes of 13 plant species, thereby providing a massive resource for the plant biology community. If these methods were modified to detect transient interactions, then the proportion of free and interacting proteins could be readily quantified. We propose that such approaches be developed to identify putative plant metabolons for future study, and those with high ratios of interacting/free enzymes be studied first.

Metabolon formation involves mechanisms by which plant metabolic regulation can be achieved (Sweetlove and Fernie, 2018).

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Given that metabolons function by channeling intermediates between the catalytic domains of sequential enzymes within the channeled microenvironment, it follows that dynamic metabolon assembly can efficiently regulate the metabolic flux within a pathway and that disassembly can mediate redirection of metabolic flux into a competing pathway(s). Given that neither the association nor disassociation of the metabolon necessarily requires large-scale energy input, such as the synthesis or degradation of proteins, nor requires additional regulatory proteins, metabolon formation was suggested to be an ideal regulatory mechanism for the rapid and frequent plant metabolic fluxes that occur in response to specific metabolic demands imposed on development or in response to a biotic challenge(s) (Møller, 2010). This mechanism has also been suggested to be an important point of metabolic control at branch points in metabolic pathways, thereby allowing the coordination of fluxes across multiple metabolic pathways (Wheeldon et al., 2016). These features are believed to be instrumental in the regulation of respiratory metabolism. This is especially the case in the glycolytic pathway (Giege et al., 2003; Graham et al., 2007) and TCA cycle (Zhang et al., 2017; Zhang and Fernie, 2018), although it is unclear whether this also applies to the mitochondrial electron transport chain remains debatable (Ramírez-Aguilar et al., 2011; Fernie et al., 2018). The plant glycolytic metabolon was suggested to be transient, depending on the energy needs and metabolic state of the cell (Giege et al., 2003; Graham et al., 2007). Similarly, the assembly or disassembly of the six enzymes constituting the purine nucleotide biosynthetic pathway into what is known as the purinosome depends on the cellular requirement of purines in humans (Zhao et al., 2013; Sweetlove and Fernie, 2018). Moreover, proteomics was utilized to define the nature of PPIs within the cluster, demonstrating that a core complex that assembles in a stepwise fashion includes the first three enzymes in the pathway, whereas further assembly of the purinosome requires the function of many auxiliary proteins (Sweetlove and Fernie, 2018). All of this evidence has been used to support the metabolic role of the purinosome, and although logical, it has the disadvantage of being circumstantial. In a very recent study, the Benkovic group (Pareek et al., 2020) provided direct proof of this fact by demonstrating that the metabolite pathway (1) is functional and (2) promotes substrate channeling (Figure 1). Although the best proof of the latter comes from isotope tracing experiments, quantitative analysis performed by ultra-high resolution mass spectral imaging provides strong support for the conclusions drawn from these analyses (Pareek et al., 2020). Currently, it is unknown whether a purinosome exists in plants; however, in plants, the pathway is mosaic in nature (Sweetlove and Fernie, 2013), divided between the cytosol and plastid, so even if it exists, it is likely to be greatly different from that found in humans.

In addition to the biosynthetic pathways of primary metabolites, a plethora of secondary metabolite pathways have been suggested to contain metabolons, with the main rationale here being either that they can avoid high levels of unstable and toxic intermediates in cells or that they can ensure high metabolic flux, thereby enabling high production of functional phytochemicals (Jørgensen et al., 2005; Møller, 2010). Although the former may be true, as we have discussed previously, arguments for the latter are less convincing (Sweetlove and Fernie, 2013, 2018; Fernie et al., 2018), with an alternative and more likely reason being, in our opinion, that metabolons act to regulate the routing of metabolic fluxes at network branch points. We provide evidence of the existence of metabolons in plant secondary metabolism in detail below. However, given that it was the earliest proven metabolon in plants following the groundbreaking work on cyanogenic glucoside biosynthesis by Møller and Conn (1980), we think it is worth mentioning in passing that the isotope dilution proof of the metabolon in this pathway has been extensively characterized by a battery of biochemical, molecular biological, and cell biological approaches (see the section Cyanogenic Glycoside Biosynthesis). Since the identification of the cyanogenic glucoside dhurrin metabolon, several enzyme complexes have been identified and found largely by PPI assays to act as metabolons (Table1). However, as mentioned above, only a few of these complexes were proved to mediate substrate channeling, such as the glycolytic pathway (Giege et al., 2003; Graham et al., 2007), TCA cycle (Zhang et al., 2017), early steps of the phenylpropanoid biosynthetic pathway (Achnine et al., 2004), and cyanogenic glucoside biosynthetic pathway (Laursen et al., 2016). Strictly speaking, metabolons have been experimentally confirmed in four biochemical pathways in plants, although it should be noted that more than one metabolon can form in some of these pathways, and the other complexes should technically be described as transient enzyme interactions.

EXPERIMENTAL EVALUATION OF PROTEIN COMPLEX ASSEMBLIES AND SUBSTRATE CHANNELING

The metabolon is a transient structural–functional substrate channel formed among sequential enzymes of a metabolic pathway and is associated with non-covalent interactions and structural elements of the cell, such as integral membrane proteins and cytoskeletal proteins (Srere, 1985; Møller, 2010). Given the transient and dynamic nature of the interactions, the detection of such enzyme complexes is more difficult compared with stable protein complexes such as those in multi-enzyme complexes. By definition, the association of the metabolon allows the metabolites to directly pass from one enzyme into the active site of the next enzyme; thus, it is very important to evaluate metabolite channeling *in vivo*. In this section, we discuss both the identification of different types of enzyme complexes and, more challengingly, the evaluation of metabolic channeling.

Identification of Transient Enzyme–Enzyme Assemblies in Protein–Protein Interaction Studies

Given their transient and dynamic association, enzyme–enzyme assemblies rapidly and efficiently assemble and disassemble. As we postulated above, in the case of substrate channels, this can be an effective approach to alter plant metabolic flux in response to specific metabolic demands. Recently developed approaches have provided more options to test PPIs, such as tandem affinity purification–MS (AP–MS), split-luciferase complementation (split-LUC) assays, bimolecular fluorescence complementation (BiFC), co-immunoprecipitation (coIP), fluorescence lifetime imaging microscopy–Förster resonance energy transfer (FLIM/FRET), yeast-two-hybrid (Y2H) assays, bioluminescence resonance energy transfer (BRET), clear-native PAGE, crosslinked MS,

Metabolic pathway	Protein–protein interactions method	Substrate channel	References
Glycolysis	2D gel, blue-native PAGE	Isotope dilution experiment	Giege et al., 2003; Graham et al., 2007
TCA cycle	AP–MS, split-LUC, Y2sH, BiFC	Isotope dilution experiment	Zhang et al., 2017
Mitochondrial electron transport chain	Protein structural evidence	Not proven	Milenkovic et al., 2017; Lobo-Jarne and Ugalde, 2018
Calvin–Benson cycle	Protein structural evidence	Not proven	Yu et al., 2020
Purine nucleotide synthesis	Proposed	Isotope dilution experiment; metabolite imaging experiments	Pareek et al., 2020
Starch synthesis	CoIP, Y2H, AP–MS, SEC, XC	Not proven	Tetlow et al., 2004; Lin et al., 2017
Polyamine synthesis	SEC	Not proven	Panicot et al., 2002
Dhurrin	SMALP, FLIM/FRET, FCS	Isotope dilution experiment	Møller and Conn, 1980; Laursen et al., 2016
Auxin	FLIM/FRET	Not proven	Kriechbaumer et al., 2017
Phenylpropanoid core pathway	FRET, coIP	Isotope dilution experiment	Achnine et al., 2004
Flavonols	CoIP, AP, Y2H	Not proven (however, metabolite protein docking models support it)	Crosby et al., 2011; Diharce et al., 2016; Fujino et al., 2018; Nakayama et al., 2019
Isoflavonoids	CoIP, BiFC, Y2H	Not proven	Dastmalchi et al., 2016; Mameda et al., 2018
Camalexin	CoIP, FRET-FLIM	Not proven	Mucha et al., 2019
Lignin	Co-sublocation, AP–MS, FRET, Y2H, BiFC, coIP	Not proven (however, metabolic modeling supports it)	Chen et al., 2011; Bassard et al., 2012; Lee et al., 2012; Gou et al., 2018; Dixon and Barros, 2019
Isoprenoids	Y2H	Not proven	Camagna et al., 2019
Bitter acids	Y2H, coIP	Not proven	Li et al., 2015
Monoterpene indole alkaloids	BiFC	Not proven	Stavrinides et al., 2015
Sporopollenin	CoIP, Y2H, FLIM/FRET	Not proven	Lallemand et al., 2013

Table 1. Enzyme Complex and Metabolon Identified in Plants

AP, affinity purification; MS, mass spectrometry; split-LUC, split-luciferase complementation; Y2H, yeast-two-hybrid assay; BiFC, bimolecular fluorescent complementation assay; coIP, co-immunoprecipitation; XC, crosslinking; SEC, size-exclusion chromatography; SMALP, styrene maleic acid lipid particle; FLIM, fluorescent lifetime measurement; FRET, fluorescent resonance energy transfer.

proximity-dependent biotin identification, and an engineered ascorbate peroxidase reporter (Roux et al., 2013; Kim and Roux, 2016; Xing et al., 2016; Lampugnani et al., 2018; Woods et al., 2019; Zhang et al., 2019, 2020a). Each of these methods has its own strengths and weaknesses in identifying PPIs, especially in regard to their specificities and sensitivities. In brief, a high-sensitivity approach will detect many interactions with a high false-positive rate in screening, whereas a low-specificity method will only detect the natively occurring interactions under specific conditions and result in a high false-negative rate. To reduce false-positive and -negative detection, several methods were suggested to provide information on *in vivo* PPIs, ideally with methods utilizing different principles of detection (Zhang et al., 2017; Bassard and Halkier, 2018).

High-Throughput Protein–Protein Interaction Detection

High-throughput methods for screening and verifying protein interactions enable the rapid characterization of a protein interaction network. The AP–MS method is one of the best developed methods, allowing both high-throughput and rapid PPI detection under native conditions, even when expressed at their native level (Zhang et al., 2019, 2020a). In this approach, the protein of interest is linked to a fluorescent protein tag (GFP), or alternatively a tandem affinity purification tag, using recombinant DNA technologies. The tagged bait protein could either be transiently transformed into plant leaves or stably transformed into plant cells to capture the protein complex by affinity to a tag-specific antibody, namely GFP-nanotrap tag or GS^{yellow} tag (Zhang et al., 2019). These protein complexes are thereby "co-immunoprecipitated" onto immobilized protein agarose beads via affinity purification prior to their detection and identification via MS. As coIP is the gold-standard assay for PPIs and MS can rapidly characterize the captured protein, AP–MS provides highly reliable and information-rich data for intra- and extra-pathway PPI networks. However, as a high number of washing steps are necessary to remove non-specific binding, this method has traditionally been unable to detect transient interactions. Recently, *in vivo* chemical crosslinking has been developed, which renders it possible to detect transiently formed

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complexes by forming covalent bonds between interacting proteins (Leitner et al., 2016). Additional technologies, such AP–MS combined with crosslinking, can preserve the crosslinked proteins during the washing steps in affinity purification (Wu and Minteer, 2015; Leitner et al., 2016). In addition, size fractionation of native protein complexes by clear-native PAGE and blue-native PAGE can also be used to establish an information-rich protein interaction network by separating the protein complex on a molecular weight gradient, followed by MS (Senkler et al., 2017; Gorka et al., 2019). Both methods have been successfully used to detect specific interactions between large protein complexes, such as the glycolytic complex of the mitochondrial outer membrane (Graham et al., 2007). More recently, co-fractionation MS was used to analyze the protein complex across 13 species (McWhite et al., 2020; Zhang et al., 2020b). However, transient PPIs cannot currently be detected by these methods, and thus, are not widely used for metabolon identification.

Y2H screening is a very high-throughput molecular biology technique used to identify putative interactions (Perkins et al., 2010). Y2H allows the screening of protein libraries of a bait protein's potential interactors as well as the identification of binary interaction pairs. The principle of Y2H is the activation of downstream reporter genes, such as the lacZ reporter gene and HISTIDINE3, by the binding of a transcription factor to the upstream activating sequence. The transcription factor is split into two separate fragments, a DNA-binding domain (BD) and an activating domain (AD), which are subsequently used in a protein-fragment complementation assay (PfCA) in the yeast nucleus. Plant cDNAs are randomly ligated into vectors containing the AD sequence, thus producing a library that represents the prey in the subsequent screen. The bait gene is recombined with the BD and co-transformed into yeast cells to screen the candidates on the basis of reporter gene activation. However, it is unclear whether the protein interactions in the yeast nucleus are similar to those in plant cells, as the method produces a high rate of false-positive detections, especially for interactions between proteins that *in planta* are localized in different subcellular locations. A further limitation is that several interactions also need co-factors for protein complex assembly. Despite these facts, this approach does have the ability to detect transient interactions (Brückner et al., 2009), as well as to identify membrane protein interactions, at least in its modified split ubiquitin-based form. Furthermore, given its scalability and accessibility, this method remains the most popular for the large-scale detection of PPIs (Brückner et al., 2009). Indeed, several recent modifications of Y2H have improved the detection of additional types of interactions, including those relating to membrane-bound, cytosolic, or extracellular proteins (Zhou et al., 2016; Zhang et al., 2017), rendering it an important primary screen for a wide range of protein types, albeit being a technique whose results need definitive validation by an alternative method.

Protein-Fragment Complementation Assays

PfCAs are assays for the identification of PPIs, which have been used to provide a simple and direct way to study PPIs both *in vivo* and *in vitro*. In these studies, the tagged protein (fluorescent protein, luciferase, or transcription factor) is split into two parts by linking with bait and prey, and reconstituted non-covalently to form a functional protein using Y2H, split-LUC assay, or BiFC. Thus, the split fragments should have low affinity for one another such that they can only be reconstructed by linked interacting proteins, i.e., the interaction of bait and prey. Despite advances in the implementation of these techniques, an important subset of transient interactions is dependent on post-translational modification events. Such interactions are often missed when PPI screening is performed by Y2H (Leitner et al., 2016). This limitation has been tackled in several different ways, including the use of native cell culture systems. Among other methods that use endogenous cell systems for PPI screening, the split-LUC assay (Varnum et al., 2017) and BiFC (Kudla and Bock, 2016; Xing et al., 2016; Zhang et al., 2020a) are common, both of which can detect transient (as well as permanent) PPIs *in vivo*. In a split-LUC assay, the PPIs of the bait and prey can bring the two separate luciferases together to transiently reconstitute an active luciferase, such as firefly luciferase, nano luciferase, or Renilla luciferase (Wang et al., 2019). Although this method is difficult to use for high-throughput interaction studies, the split-LUC assay is particularly powerful for pinpointing transient and reversible PPIs under native conditions. The intensity of the luciferase activity is proportional to the strength of the PPIs, with higher luciferase signals indicating close or direct protein interactions and lower signals indicating weak interactions or interactions within a protein complex (Zhou et al., 2018). For this reason, this method is widely used for metabolon investigations. In addition, BiFC, which is based on the assembly of split fluorescent protein fragments, is widely used to detect protein interactions. This technology allows the detection of the fluorescent signal with localization information within cells by confocal microscopy (Kudla and Bock, 2016; Xing et al., 2016). Similarly, in the split-LUC assay, the intensity of the fluorescence emitted is proportional to the strength of the interaction, with stronger fluorescence levels indicating close or direct interactions and lower fluorescence levels suggesting interactions within a complex. Therefore, through the visualization and analysis of the intensity and distribution of fluorescence in these cells, one can identify both the location and interacting partners of target proteins. Because of these advantages, this method is widely used to evaluate protein complexes in plants. However, this approach cannot monitor dynamic PPIs due to the irreversibility of the reconstituted fluorescent protein (Kudla and Bock, 2016; Xing et al., 2016). The reconstituted fluorescent protein can also increase the false-positive rate. Thus, in addition to split-LUC, dynamic methods, such as BRET and FRET, should be used to decrease the false-positive rate.

Biophysical Analysis of Interactions

The formation of a dynamic multi-protein complex is characterized by reversible assembly, and different complexes exhibit different binding affinities (Zhang and Fernie, 2020). The cooperativity and multi-valent binding of these components can be characterized *in vitro* by a range of different biophysical analyses. Biomolecular interactions, including PPIs, can be easily, rapidly, and precisely quantified by microscale thermophoresis (MST) (Wienken et al., 2010). Based on the identification of temperature-induced changes in the fluorescence of a probe as a function of the level of a non-fluorescent ligand, MST measures the movement of molecules on microscopic temperature gradients and discovers changes

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in their hydration shell, size, or charge to evaluate their binding affinities. The measured changes in fluorescence come from two sides, one based on a temperature-related intensity change of the fluorescent target and the other based on the directed motion of a molecule in the aforementioned microscopic temperature gradient. When a temperature gradient is applied, the binding affinity of a protein complex can be quantified. This technique also has the advantage of allowing the determination of interactions directly in solution without the need for surface immobilization (Jerabek-Willemsen et al., 2011). However, the hydrophobicity of the fluorescent-labeled probe may result in unspecific binding. With these interactions being highly sensitive to changes in temperature, the detection of false signals is a problem. By contrast, coIP has been reported to be the most reliable assay to detect putative PPIs, especially when it is used to detect endogenous proteins as opposed to tagged overexpressed proteins. In such cases, the prey and interacting partners are isolated by a specific prey antibody and identified by western blotting. Indeed, coIP has been widely used for binary PPIs of protein complexes such as the glycolytic complex in yeast and humans (Puchulu-Campanella et al., 2012; Araiza-Olivera et al., 2013). However, this method is mostly used for binary interaction pairs, rather than broad screening. The use of a highly specific prey antibody represents a very important mechanism to decrease the identification of false-positives. However, the method, when coupled with MS, does detect both direct and indirect interactions, and therefore, has found great utility in the identification of enzyme–enzyme complexes (Free et al., 2009).

Dynamic Analysis of Protein–Protein Interactions

Given that the conditional transient association and disassociation of metabolons allow the plant to rapidly, flexibly, and coordinately reconfigure its metabolism in response to different environmental conditions, it is important to characterize this dynamic behavior. Therefore, the detection of these transient and reversible PPIs under their native conditions is desirable. As mentioned above, the split-LUC assay can provide important information on the dynamics of protein complexes. However, the intensity of luciferase activity is not only dependent on the PPIs but also on the uptake of its substrate. Given that the majority of split-LUC assays are set up for transient experiments using different bait and prey expression levels in plant protoplasts or tobacco leaves, the dynamics of PPIs cannot be readily detected by this method. BRET (Machleidt et al., 2015) and FRET (Grünberg et al., 2013) were developed to detect protein interactions in living cells. For this purpose, one protein is fused to NanoLuc luciferase or mCitrine and the other protein to Halotag or mCherry. After introducing the fusion proteins via transgenesis, BRET and FRET between the two proteins are measured (Grünberg et al., 2013; Machleidt et al., 2015; Goyet et al., 2016). These approaches were demonstrated to have the additional benefit of highlighting transient, membrane-bound, and extracellular protein interactions, as they can detect interactions on the basis of physical distance (Acuner Ozbabacan et al., 2011). Measurements obtained by these methods thereby provide a non-invasive means by which to visualize the spatiotemporal dynamics of interactions between protein partners *in vivo*. In addition, the use of confocal laser scanning microscopy may enable the direct imaging of metabolons following the stable or transient expression of target proteins as fluorescent-fusion proteins or as immunologically tagged proteins (Winkel, 2004; Jørgensen et al., 2005). Direct visualization of metabolons, which allows assessment of their formation by sequential incorporation of their component polypeptides, can be achieved by atomic force microscopy (Bayburt and Sligar, 2002) or FLIM/FRET (Laursen et al., 2016). As both methods measure the efficiency of energy transfer from the donor to acceptor, the distance of PPIs plays an important role in their efficiency. Given that small changes in distance can be easily detected, false-negative results may occur given the large distance between bait and prey. In addition, these methods have been utilized to counteract the problem of false-positive PPIs identified by other methods.

Weak and Strong Transient Interactions

Transient protein interactions can be further subdivided into weak and strong interactions. Strong transient interactions are mostly characterized by substrate channeling, whereas weak transient interactions tend to be involved in signaling. One of the most popular methods for identifying transient complexes is combining chemical crosslinking with MS, which affords the possibility of freezing transiently formed protein complexes by inducing *in vivo* covalent bond formation between their interacting components (Wu and Minteer, 2015). Moreover, new methods, such as proximity-dependent biotin identification and an engineered ascorbate peroxidase reporter, enable the isolation and characterization of enzyme–enzyme assemblies in which adherence between the different subunits is governed by a weak transient interaction (Roux et al., 2013; Kim and Roux, 2016; Woods et al., 2019). However, these methods normally detect very weak interactions found in the signal transduction components of cells, and as such are not widely used to study enzyme–enzyme assemblies.

In Silico Methods for the Prediction of Protein Interactions

Although experimental approaches have recently provided a torrent of protein interaction information, a variety of *in silico* methods have been developed to predict the interactions based on a range of different approaches, including sequence-based approaches, structure-based approaches, chromosome proximity, gene fusion, and gene expression-based approaches. Among them, the structure-based method uses a high-throughput docking algorithm that can distinguish putative interactions from false interactions (Dong et al., 2019). The docking algorithm of Dong et al. (2019) can predict the binding interface, evaluate the compatibility of the interface with an interface coevolution-based model, and provide a confidence score for the interaction. Similarly, three-dimensional structural information has been used to predict PPIs with accuracy and coverage that are far superior to predictions based on non-structural evidence (Zhang et al., 2012). In keeping with this development, several studies have focused on ensemble methods to integrate different PPI methods to enhance the reliability of interaction lists (Szklarczyk et al., 2018). For instance, the database STRING v11 collects, scores, and integrates all publicly available experimental sources of PPI information and allows the comparison of these with computational predictions (Szklarczyk et al., 2018).

The Use of Multiple Methods to Reliably Identify PPI Networks

As already discussed, no method is perfect in detecting all putative interactions without producing additional false-positive or missing false-negative interactions. All these methods tend to generate many false-positive results, and therefore, special caution needs to be exercised in the interpretation of their results, in particular with regard to the use of proper negative controls (Kudla and Bock, 2016; Xing et al., 2016). This fact renders the identification and screening of novel enzyme–enzyme interactions complicated. Additionally, given the relatively high false-positive and -negative rates of these assays, evidence from multiple independent approaches are required to accurately call the presence of enzyme–enzyme assemblies (Zhang et al., 2017). The use of multiple techniques greatly aids in the reduction of false-positive results, thereby improving the reliability of the data. However, these techniques require special equipment and expertise, and their use is often limited to highly specialized research groups. When the purpose of an experiment comes to the screening of interacting protein pairs or protein complexes within a large set of enzymes, it becomes increasingly complicated to obtain reliable results. Therefore, the use of multiple techniques is essential to avoid not only false positives but also false negatives. Given the transient nature of non-permanent enzyme–enzyme assemblies, their associations are most likely dependent on the microenvironments in which the enzymes find themselves. As such, the use of a single method may provide an inappropriate microenvironment leading to false-negative results. Conversely, problems arise in the integration of results from multiple techniques and in setting an appropriate threshold at which to declare a protein pair or multiple proteins as interacting with one another (Zhang et al., 2017). In the screening of PPIs within the plant TCA cycle, a combination of three conventional methods, namely Y2H, split-LUC complementation, and AP–MS, has been used (Zhang et al., 2017, 2018). All three of these approaches provide information on *in vivo* PPIs, yet the principle of detection in all instances is different. Importantly, however, they produce semi-quantitative scores that can be statistically combined to generate a single reliability score. This approach successfully identified expected and novel components of the plant TCA-cycle metabolon (Zhang et al., 2017), and as such provides a robust framework for the screening of physiologically relevant enzyme–enzyme assemblies. When the purpose of an experiment is to screen interacting protein pairs or protein complexes within a large set of enzymes, it becomes increasingly complicated to obtain reliable results. The use of multiple techniques is essential in avoiding not only false positives but also false negatives. Given the transient nature of metabolons, their association is most likely dependent on the microenvironments of the enzymes. A single method may provide an inappropriate microenvironment, leading to false-negative results. Nevertheless, problems then arise in the integration of results from multiple techniques and in setting a proper threshold at which to declare that a protein pair interacts.

BIOCHEMICAL METHODS OF EVALUATING SUBSTRATE CHANNELING

Although the aforementioned approaches represent the state of the art in detecting protein–protein assemblies, the specific detection of metabolons requires stricter proof than that commonly provided (Zhang et al., 2017; Fernie et al., 2018; Sweetlove and Fernie, 2018). Identification of PPIs that include sequential enzymes certainly provides a plethora of protein complexes that may mediate substrate channeling. However, before describing such enzyme associations as metabolons, proof of substrate channeling is required, such as that provided for glycolytic and TCA-cycle metabolons as mentioned above (Giege et al., 2003; Graham et al., 2007; Wu and Minteer, 2015; Zhang et al., 2017). The quantitative examination of metabolic channeling has largely focused on the indirect measurements of the effects of the absence or presence of pathway intermediates in the bulk phase. These measurements all indicate the access of the metabolites to the bulk phase or, more correctly, the access of the bulk phase to the active sites of the enzymes under study. There are five standard biochemical methods to evaluate substrate channeling, namely (1) isotope dilution and enrichment studies, (2) transient time (τ) analysis, (3) resistance to a competing side reaction, (4) resistance to a reaction inhibitor, and (5) enzyme-buffering analysis of channeling (Fernie et al., 2018; Sweetlove and Fernie, 2018). Here, we discuss each method, although most of these methods have not been used in plants.

Labeling methods have been successfully used to evaluate the occurrence of substrate channeling in the glycolytic cycle (Graham et al., 2007), TCA cycle (Zhang et al., 2017), early steps of phenylpropanoid biosynthesis (Panicot et al., 2002), and cyanogenic glucoside biosynthesis (Møller and Conn, 1980). The principle of the isotope dilution method is that labeled metabolite pools (A*) are incubated with the protein complex, followed by the addition of unlabeled metabolites (B) to ascertain the presence or absence of substrate channeling. The ratio of labeled (C*) and unlabeled products provides quantitative information on channeling. In a channeled metabolon, labeled intermediates will not enter the bulk phase and the labeled product will not be diluted by additional unlabeled metabolites. Thus, the unlabeled products will not be detected in a saturated enzyme assay. By contrast, the labeled intermediate will come into contact with the bulk phase in an unchanneled system. In this case, unlabeled products will be produced and the ratio between labeled and unlabeled products will decrease. Given that in most instances, enzymes exist both in their free states and in assemblies due to their transient nature, the results are often not so clear cut.

However, this approach was used in 1980 to successfully identify the dhurrin metabolon (Møller and Conn, 1980). Early biosynthetic studies using microsomal preparations from etiolated *Sorghum bicolor* seedlings and the administration of dual stable isotope-labeled precursors revealed tight substrate channeling (Møller and Conn, 1980; Halkier et al., 1989). The approach was subsequently used to prove the presence of a glycolytic metabolon in *Arabidopsis* mitochondria (Giege et al., 2003; Graham et al., 2007). In this study, labeled glucose was incubated with purified mitochondria, unlabeled G6P, F6P, F16BP, dihydroxyacetone phosphate, and glyceraldehyde-3-phosphate were subsequently added separately, and the proportional labeling of pyruvate was followed over time. The results indicated that a protein complex of phosphofructokinase (PFK), aldolase, triosephosphate isomerase, and

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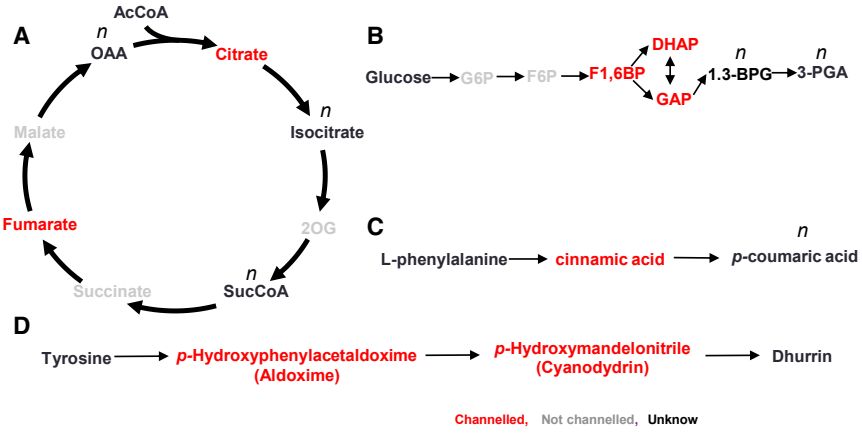

Figure 2. Characterized Metabolon in Plants.

(A) Metabolon of the TCA cycle (Zhang et al., 2017).

(B) Glycolysis metabolon (Graham et al., 2007).

(C) Early steps of phenylpropanoid biosynthesis (Achnine et al., 2004).

(D) Cyanogenic glucoside biosynthetic pathway (Laursen et al., 2016). The red metabolites are channeled. The gray metabolites are unhandled. The channel information of black metabolites is unclear, denoted as *n* in **(A)** to **(C)**.

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) formed a substrate channel with 100% of intermediates being channeled, while a protein complex of hexokinase, phosphoglucose isomerase, and PFK was not fully channeled with only 38% of G6P and 63% of F6P being channeled. In addition, the undetected labeled intermediates in the bulk phase are indicative of full channeling, whereas the increased concentration of labeled intermediates in the bulk phase represents leaky channeling. Thus, the concentration of labeled intermediates can also provide information on the extent of channeling. In our own former research, ^{13}C-labeled pyruvate or glutamate was fed to isolated potato tuber mitochondria until the accumulation of ^{13}C in downstream TCA-cycle metabolites reached an isotopic steady state (Zhang et al., 2017). After adding unlabeled TCA-cycle metabolites, the dilution effects on labeling were monitored at different time points. When the purified mitochondria were fed with pyruvate or glutamate, the TCA cycle was linearized by inhibiting succinate dehydrogenase with malonate or aconitase with fluoroacetate to eliminate complications in the interpretation of labeling patterns caused by multiple turns of the cycle. In metabolite measurements, diluted 2-oxoglutarate, succinate, and malate were detected, whereas undiluted citrate or fumarate was not observed, revealing a channeled flux from fumarate to malate, but not from 2OG or succinate to citrate. Although the demonstration of this plant TCA-cycle metabolon is important, it is interesting to note that this finding is, at least partially, different from results reported for animals and microbes. Metabolons have also been postulated to be an important part of phenylpropanoid metabolism; however, in 2004, substrate channeling between phenylalanine ammonia lyase (PAL) and cinnamate-4-hydroxylase (C4H) was proved by another variant in isotope dilution experiments. In their study, Achnine et al. (2004) revealed that in *Nicotiana tabacum* a significant amount of the [^3H]-*trans*-cinnamic acid converted from [^3H]-l-Phe did not equilibrate with exogenously added [^{14}C]-*trans*-cinnamic acid, suggesting that it is rapidly channeled through the C4H reaction to 4-coumaric acid. To better illustrate how such data are interpreted, we provide both pathway maps and cartoons of the experimental data emanating from the study of the four different pathways in Figure 2.

Given that they have not been extensively used in plants, we refer readers interested in the methods of transient time (τ) analysis, resistance to a competing side reaction, and resistance to a reaction inhibitor to earlier reviews on the metabolon (Wu et al., 2015; Fernie et al., 2018; Sweetlove and Fernie, 2018). Although it has not been extensively used in plant research, we discuss here the enzyme-buffering method, as it is well grounded in theory and well supported by structural biological evaluations. Furthermore, it is broadly applicable; indeed, NADH buffering analysis is typically applied to follow the channeling of NADH, which assesses whether the second enzyme of a complex can use bound, as well as free NADH, and is based on the comparison of the reaction velocities following dramatic decreases in the size of the free NADH pools as represented in the scheme of Spivey and Ovádi (1999) (Figure 3). If the second enzyme is not able to utilize bound NADH, the system is essentially buffering the NADH added to it, hence the name (Srivastava and Bernhard, 1986, 1987). For example, an early experiment that demonstrated the direct transfer of NADH from liver GAPDH to alcohol dehydrogenase was used to evaluate substrate channeling (Srivastava and Bernhard, 1984), and these authors reported that a series of such transfers can occur between dehydrogenases. However, this method can only be used between dehydrogenases with alternative stereospecificity for NADH.

To summarize, there are three types of sequential enzymes, namely the fully channeled complex, the partly channeled complex, and the absence of substrate channeling (Wheeldon et al., 2016). The evidence of fully channeled metabolons is usually clear compared with that of partly channeled protein complexes. Leaky channeling can be difficult to strictly evaluate and may be dependent on various factors, including cascade kinetics, substrate concentrations, the system pH, and the physical structure of the complex. Moreover, channeling may be difficult to quantify due to the sensitivity and response time of kinetic measurements and changes in cascade structure induced by experimental testing (Spivey and Ovádi, 1999; Wheeldon et al., 2016). Finally, it is important to note that for all the aforementioned methods of detecting substrate channeling, identification is provided by indirect experimental evidence. Thus, multiple methods should be used to support a claim of channeling such as structural evidence and protein complex association. During the writing of the initial draft of this review, a novel technique relating to the study of the human purinosome has been proposed, namely high-resolution mass spectral imaging. Pareek et al. (2020) used this technique in combination with isotope tracer experiments to confirm the functionality of the enzymes of the purinosome, the presence of

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channeling, and the enhanced local concentrations of the metabolic intermediates of the pathway within the purinosome. This powerful approach suggests that this technique should, whenever possible, be adopted in studies of putative metabolons. Interested readers are referred to the excellent recent review by van Hove et al. (2010). Another technology that merits discussion is *in situ* cryoelectron tomography (Asano et al., 2016), which allows the visualization of enzyme complexes in their native environments and may give good insights on structural channeling. One would anticipate that its utility in the study of enzyme–enzyme assemblies will dramatically increase in the coming years.

EVALUATION OF METABOLONS IN PRIMARY METABOLIC PATHWAYS

Plants obtain energy almost entirely by photosynthesis and produce numerous important compounds, including primary and secondary metabolites. Primary metabolites are those directly involved in normal growth, development, and reproduction of the organism. They are typically key components of normal physiological processes and form during the growth phase as a result of energy metabolism, such as the Calvin–Benson cycle, glycolysis, and the TCA cycle. Therefore, metabolic fluxes rapidly fluctuate to the environments in which plants are exposed. They achieve this by several regulatory mechanisms, including the dynamic formation of metabolons. In plants, both glycolysis and the TCA cycle are well documented to form protein complexes, while the constituents of the mitochondrial electron transport chain (Fernie et al., 2018), Calvin–Benson cycle (Yu et al., 2020), photorespiration (Douce et al., 2001), starch metabolism (Tetlow et al., 2004; Lin et al., 2017), polyamine synthesis (Panicot et al., 2002), and *S*-adenosyl methionine formation (Zeng et al., 2020) were also suggested to form metabolons; however, to date, these claims remain unsupported with reliable proof that the observed enzyme–enzyme assemblies support substrate channeling. Here, we provide a brief overview of the current understanding of each of these enzyme–enzyme assemblies, paying most attention to glycolysis and the TCA cycle.

Glycolysis

Given that the first experimental evidence, that is, independent pools of glycolytic intermediates in *Escherichia coli* and rats, provided controversial and indirect proof for the subcompartmentation of metabolic processes (Connett et al., 1972; Lynch and Paul, 1983; Fernie et al., 2018), assemblies of glycolytic enzyme complexes have been extensively assumed. Considerably stronger proof for the association of glycolytic enzymes has been provided such as direct evidence of specific assembly between sequential pairs of glycolytic enzymes (Connett et al., 1972; Masters, 1981; Fernie et al., 2018; Sweetlove and Fernie, 2018) or interaction of glycolytic enzymes and actin (Morton et al., 1977; Masters, 1981; Fernie et al., 2020) and complexes of all glycolytic enzymes (Connett et al., 1972; Masters, 1981). Srere (1987) first provided evidence of interactions between sequential enzymes of GAPDH and aldolase to support glycolytic enzyme assembly. Following this, a range of experimental methods, including kinetic studies, the polarization of fluorescence, affinity electrophoresis, and active enzyme centrifugation, were used to investigate these interactions (Fernie et al., 2018). A particular breakthrough was the discovery of the association of hexokinase with the mitochondrial outer membrane. There are several advantages of this sublocation, including energy efficiency in that it provides immediate access to the ATP being generated by mitochondria, leading to the suggestion that metabolic channeling occurs with associated kinetic and regulatory benefits (Srere, 1987). A milestone proteomic study reported that additional glycolytic enzymes associated with the outer membrane of mitochondria from *Arabidopsis* to establish substrate channeling (Giege et al., 2003), with a similar report of enzyme association in humans (Puchulu-Campanella et al., 2012). Furthermore, a large macromolecular complex associated with mitochondria has been proved by employing both enzyme assays and blue-native SDS–PAGE as well as coIP of proteins with an anti-enolase antibody in yeast (Brandina et al., 2006; Araiza-Olivera et al., 2013).

In addition to the protein association evidence, subsequent experiments of isotope dilution and NADH channeling analysis were used to evaluate substrate channeling (Giege et al., 2003). As mentioned above, the glycolytic substrates [^{13}C]-glucose and [1-^{13}C]-fructose-1,6-bisphosphate were supplied to isolated mitochondria, demonstrating that the complete glycolytic cascade was present and active in this fraction. Further research, this time on potato mitochondria, showed that this association was dynamic in that the inhibition of respiration by potassium cyanide led to a proportional decrease in the association of glycolytic enzymes with mitochondria, whereas stimulation of respiration enhanced this association (Fernie et al., 2018). This proof of dynamism, similar to that observed in the cyanogenic glycoside pathway, when taken alongside the evidence of channeling, is suggestive of a physiologically important interaction. Indeed, these findings are highly important in establishing the function of metabolons, and their relative paucity remains a bone of contention to metabolic biologists who doubt their importance. In the case of glycolysis, however, this was clearly demonstrated. Indeed, further studies demonstrated that the association is mediated by the outer mitochondrial membrane porin protein VDAC, which anchors the glycolytic enzymes to the membrane (Graham et al., 2007).

The TCA Cycle

Several early experiments have used different approaches to support the concept of the organization of the TCA-cycle enzyme complex (Srere et al., 1973), with a fresh impetus provided after Srere first termed such complexes metabolons (Srere, 1987). Srere et al. (1973) demonstrated that an immobilized pairing of malate dehydrogenase and citrate synthase had a kinetic advantage over the free enzymes. Moreover, as shown for glycolysis, advancements in fluorescence-based cell biology and proteomics facilitated the study of pathway-wide interactions in the TCA cycle. For example, a comprehensive characterization of the interactome between *Bacillus subtilis* enzymes revealed interactions between six consecutive enzymes of the TCA cycle. Additionally, in a more recent study, Wu and Minteer (2015) coupled *in vivo* crosslinking and MS and demonstrated that structural models of TCA-cycle enzyme complexes are consistent with substrate channeling via electrostatic retention of the channeled metabolite on charged domains of the enzyme

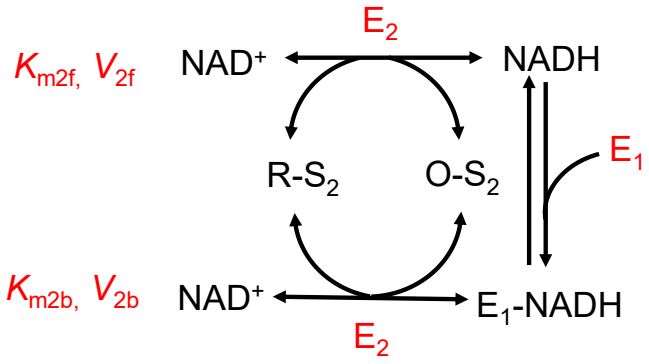

Figure 3. Enzyme-Buffering Analysis of Channeling.
This approach is typically applied for following the channeling of NADH, which assesses whether the second enzyme of a complex can use bound, as well as free NADH, and is based on the comparison of reaction velocities, following dramatic decreases in the size of the free NADH pools as represented in the scheme. If the enzyme cannot utilize bound NADH, then the system is essentially only buffering the NADH added to it, hence the name. Modified from Zhang et al., 2020a.

surfaces (Wu and Minteer, 2015; Wu et al., 2015). Interestingly, crosslinking revealed interactions between all eight enzymes of the cycle. Using distance constraints derived from crosslinking, two possible models of the malate dehydrogenase–citrate synthase–aconitase complex were proposed (Zhang and Fernie, 2018). Various PPI and isotope-labeling approaches (described above) have also been used to successfully provide direct or indirect evidence for substrate channeling in the TCA cycle (Zhang et al., 2017; Fernie et al., 2018).

The Mitochondrial Electron Transport Chain

Mitochondrial electron transport chain (mETC) complexes are well-reported stable multi-subunit protein complexes that have been subject to considerable research effort given the importance of mETC dysfunction in humans (Milenkovic et al., 2017). As in other organisms, individual mETC complexes can dynamically organize into supercomplexes in plants (Eubel et al., 2003; Ramírez-Aguilar et al., 2011). Although some species-specific variations between these supercomplexes have been reported, mETC supercomplexes are now accepted by the scientific community. The plant mETC is more divergent than that in other species in terms of size and the respiratory pathway used, which has not been identified in microbial or mammalian systems until recently (Del-Saz et al., 2018). Studies on the structures of proton translocating complexes (complexes I, II, III, and IV), as well as mobile electron carriers (ubiquinone and cytochrome *c*), suggested the possible substrate channeling of proton translocation to generate a proton-motive force for ATP synthesis (Milenkovic et al., 2017; Lobo-Jarne and Ugalde, 2018; Toleco et al., 2020). However, the evidence of substrate channeling in mETC supercomplexes remains hotly debated, with groups discussing whether structural evidence is consistent with the occurrence of this phenomenon (Althoff et al., 2011; Dudkina et al., 2011; Letts et al., 2016; Sousa et al., 2016). Given that we have extensively reviewed this evidence recently (Fernie et al., 2018; Zhang and Fernie, 2020), we state here that we are of the opinion that robust evidence for the channeling of electron carriers between respiratory complexes is currently lacking.

PHOTOSYNTHESIS, PHOTORESPIRATION, AND SYNTHESIS OF STARCH, SUCROSE, PURINE NUCLEOTIDE, POLYAMINE, AND *S*-ADENOSYLMETHIONINE

Having discussed the respiratory pathways in detail above, we now discuss other prominent pathways of primary metabolism for which the existence of metabolons has been postulated. Given the microcompartment of chloroplast stroma, a multi-enzyme complex of the Calvin–Benson cycle catalyzing carbon fixation has been postulated. Indeed, the recently reported cyanobacterial GAPDH/CP12/phosphoribulokinase (PRK) structure provides solid evidence for enzyme–enzyme assembly in this complex (Yu et al., 2020). Earlier studies have proposed the potential multi-enzyme complex of ribose-phosphate isomerase, PRK, ribulose-bisphosphate carboxylase/oxygenase, phosphoglycerate kinase (PGK), and GAPDH within a stable 900-kDa supercomplex (Suss et al., 1993; Graciet et al., 2004; Yu et al., 2020). The interaction between aldolase and GAPDH was identified by several methods in pea chloroplasts (Anderson et al., 1995), whereas phosphoribose isomerase, PRK, PGK, GAPDH, and fructose 1,6-bisphosphate were co-localized in immunolocalization studies in the algae *Chlamydomonas reinhardtii* (Suss et al., 1995). However, proof of direct protein interaction and substrate channeling is currently lacking in the Calvin–Benson cycle. Similarly, in the central photorespiratory pathway, possible interactions between glycolate oxidase, catalase, Ser-glyoxylate and Glu-glyoxylate aminotransferase, hydroxypyruvate reductase, and malate dehydrogenase have been postulated to occur in peroxisomes (Zhang and Fernie, 2020). Analysis of the *in vivo* assembly and dynamics of these multi-enzyme complexes in peroxisomes may reveal additional regulatory mechanisms for photorespiration (Smirnoff, 2019). Moreover, the possibility of metabolite channeling among the components of the glycine decarboxylase system also remains unclear, suggesting that further research on the Calvin–Benson cycle is needed. In addition, starch branching enzymes (SBEs; SBEI, SBEIIa, and SBEIIb) were suggested by coIP analysis to assemble into an SBEI/SBEIIb/starch phosphorylase protein complex (Tetlow et al., 2004; Lin et al., 2017). However, these findings await further studies to test their physiological relevance. One potential metabolon we can exclude is that proposed to occur on the basis of the crystal structure of an enzyme from the sucrose synthesis pathway, that is, between sucrose phosphate synthase and sucrose phosphate phosphatase, since we recently demonstrated that these proteins did not interact with one another (Zhang et al., 2020a). It has also been proposed that *de novo* purine biosynthetic enzymes cluster near mitochondria and microtubules to form dynamic multi-enzyme complexes, referred to as purinosomes, under circumstances of high purine demand in human cells (Zhao et al., 2013; Chan et al., 2015; Pedley and Benkovic, 2017; Sweetlove and Fernie, 2018). Remarkably, under cellular circumstances that lead to high demand for purines, the enzymes of de novo purine biosynthesis cluster into multi-enzyme complexes that have been dubbed purinosomes (Pedley and Benkovic, 2017). Recent research supports the substrate channel of the purinosome by high-resolution mass spectral imaging (Pareek et al., 2020). Moreover, given that a

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portion of the pathway is plastidial in *Arabidopsis*, any such channel would likely be very different in plants. In the case of the polyamine metabolon, strong biochemical, genetic, and cell biological support was provided, but again there was no proof of substrate channeling (Panicot et al., 2002), whereas the evidence for the most recently claimed metabolon, that of the *S*-adenosylmethionine cycle in peach, is even less convincing (Zeng et al., 2020). Taken together, these studies show that there is evidence for enzyme–enzyme assembly in the majority of cases but none, as yet, for substrate channeling. Although all are worthy of further study, with the exception of the specific route of sucrose biosynthesis discussed above, it is imperative that convincing evidence is provided to support substrate channeling. Until this evidence is provided, one should refrain from using the term ''metabolon'' when describing them.

EVALUATION OF METABOLONS IN SECONDARY METABOLIC PATHWAYS

Plants produce various secondary metabolites, which play important roles in plant defense and human health as medicines, flavorings, and pigments (Obata, 2019). Secondary metabolite biosynthesis is believed to be largely reliant on transcriptional regulation (De Geyter et al., 2012; Chezem and Clay, 2016); however, substrate channeling is also recognized as a mechanism to control the production of specialized metabolites. For example, the dynamic association of the dhurrin biosynthetic metabolon allows dhurrin to function in the defense of plants to the chewing insect (Laursen et al., 2016). Based on our argument, the dynamic formation of the metabolome can also be an effective mechanism of channeling resources into the production of a defense compound under conditions of urgent need. We discuss here several pathways for which metabolons have been proposed, focusing on cyanogenic glycoside biosynthesis and phenylpropanoid metabolism, which are by far the best characterized, as well as detailing the evidence (or lack thereof) for the presence of metabolons in camalexin (Mucha et al., 2019) and bitter acid (Li et al., 2015) as well as auxin (Kriechbaumer et al., 2017), isoprenoid (Camagna et al., 2019), alkaloid (Zhang et al., 2018), and wax (Lallemand et al., 2013) biosynthesis.

Cyanogenic Glycoside Biosynthesis

Within plant secondary metabolism, the sorghum metabolon of dhurrin biosynthesis is the best characterized metabolon (Laursen et al., 2016). This is perhaps not surprising since, as we discussed above, it has been studied for over 40 years. In this metabolon, two sequential enzymes, cytochrome P450s (CYP79A1 and CYP71E1) and a UDP-glucosyltransferase (UGT85B1), are assembled in a complex, which converts tyrosine via aldoxime into cyanohydrin. Cyanohydrin is unstable in the cytosol, and thus, is rapidly converted into a stable cyanogenic glucoside by a UGT85 family glucosyltransferase in cyanogenic glucoside synthesis (Jones et al., 1999; Møller, 2010). For this pathway, a wide range of biophysical and cell biological proof of the protein interaction (Laursen et al., 2016) and an evaluation of substrate channeling (Møller and Conn, 1980) provide exquisite evidence that dhurrin biosynthesis is channeled (Jørgensen et al., 2005). In an elegant approach, the FLIM/FRET method was used to monitor protein interactions among individual enzymes and to rebuild the possible structure of the dhurrin metabolon in living cells. Given that this metabolon appears to anchor onto the ER membrane, the formation of the transient metabolon is sensitive to the lipid environment and dependent on the concentration of negative charges of the lipid headgroups. Moreover, metabolon formation enhances the production of intermediates *in vitro*, even when the last enzyme does not catalyze dhurrin production due to the lack of UDP-glucose, suggesting the structural advantage of metabolon (Laursen et al., 2016).

Phenylpropanoid Pathway

Phenylpropanoids, which include flavonoids, sinapate esters, stilbenes, and lignins, play important roles in acclimation to abiotic and biotic environments, as well as acting as pigments, signaling molecules, and structural components (Tohge et al., 2013). These metabolites need to accumulate rapidly in response to fluctuating environments as well as growth and developmental cues. Three key enzymes, PAL, cinnamate 4-hydroxylase (C4H), and 4-coumaroyl-CoA ligase (4CL), convert phenylalanine to *p*-coumarolyl-CoA. The complex of these enzymes was recently demonstrated in several species by PPI methods (Nakayama et al., 2019). Importantly, substrate channeling between PAL and C4H has previously been demonstrated in isotope dilution experiments. In these experiments, a significant proportion of the [^{3}H]-*trans*-cinnamic acid converted from [^{3}H]-l-Phe did not equilibrate with exogenously added [^{14}C]-*trans*-cinnamic acid, suggesting rapid channeling through the C4H reaction to 4-coumaric acid (Achnine et al., 2004). Flavonoids are derived from *p*-coumaroyl-CoA via sequential enzymes, which have been proved to form a protein complex in several species, including rice, snapdragon, torenia, and soybean (Crosby et al., 2011; Fujino et al., 2018; Nakayama et al., 2019). These protein complexes associate with the ER membrane for the efficient production and temporal/spatial distribution of flavonoids. Using structural modeling, the substrate channeling of a flavonoid compound between dihydroflavonol-4-reductase (DFR) and leucoanthocyanidin reductase (LAR) was suggested for the opening of the DFR active site, giving rise to the facilitated diffusion of the DFR product toward LAR cavity (Diharce et al., 2016). PPIs for this pathway have been well documented in two recent reviews (Nakayama et al., 2019; Obata, 2019); however, there is currently no direct proof of substrate channeling in the latter steps of this pathway. Although several studies demonstrate PPIs in this pathway (Panicot et al., 2002; Crosby et al., 2011; Fujino et al., 2018; Nakayama et al., 2019), as well as their paralogs in isoflavonoid metabolism (Dastmalchi et al., 2016; Mameda et al., 2018), there is relatively little overlap in the exact identity of the interactions observed. In addition, C4H and *p*-coumaroyl ester 3′-hydroxylase (C3′H) of the lignin biosynthesis cascade, which operates downstream of the core phenylpropanoid pathway, were associated with each other, thereby forming a protein complex in *Arabidopsis* and *Populus* (Chen et al., 2011; Bassard et al., 2012). This complex was further confirmed to interact with a scaffold protein of membrane steroid-binding proteins (Gou et al., 2018). However, there was no direct protein interaction of this complex with C4H, C3′H, and F5H and no demonstration of substrate channeling (Gou et al., 2018). Therefore, further substrate

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channeling evidence is required to substantiate claims of a lignin metabolon and, for the same reason, for the recently characterized enzyme–enzyme assembly of lignan metabolism (Ono et al., 2019). Circumstantial evidence for a lignin metabolon has been provided by a computation analysis of the network of lignin biosynthesis (Lee et al., 2012), suggesting that, for this pathway at least, such studies would likely be worthwhile in proving substrate channeling.

Camalexin, Bitter Acid, Auxin, Alkaloid, and Wax Biosynthesis

Having covered cyanogenic glucosides and phenylpropanoids in detail, we complete this review by describing several other secondary metabolic pathways for which metabolons have been postulated. Late last year, a "metabolon" was described for the formation of phytoalexin and camalexin (Mucha et al., 2019). Although this study provided elegant proof via FRET of the enzyme–enzyme assembly and improved biochemistry of the assembled enzymes as opposed to their operation in the isolation and tight interaction of the constituent enzymes, its evidence of substrate channeling still falls short of our definition of a metabolon. This is not to discredit the work, which is a truly excellent characterization of an enzyme–enzyme assembly; we merely point out that it, like so many other studies, in our opinion misused the term metabolon. Similarly, the evidence provided by Li et al. (2015) for their enzyme–enzyme assembly involved in bitter acid formation is strong in that it comes from multiple experiments, but it does not provide any evidence for substrate channeling. A similar critique applies to studies claiming the existence of an auxin metabolon (Kriechbaumer et al., 2017) and recent studies indicating the protein interaction between *Arabidopsis* GGPP synthase (GGPS) and phytoene synthase, which are both important in isoprene biosynthesis (Camagna et al., 2019). Moreover, a study of protein complexes involved in plant alkaloid biosynthesis used BiFC to detect the interaction between strictosidine glucosidase and tetrahydroalstonine synthase in producing and consuming the toxic strictosidine aglycone, respectively (Stavrinides et al., 2015). In addition, in the morphine biosynthetic pathway of opium poppy, the fusion enzyme of a cytochrome P450 and oxidoreductase efficiently produces morphinan, potentially via metabolite channeling (Winzer et al., 2015). Although these two studies point to the possibility of substrate channeling in alkaloid biosynthesis, direct evidence of substrate channeling is missing (Stavrinides et al., 2015). Finally, as the major component of the wax coating the outer surface of pollens and spores, sporopollenin is synthesized by four ER co-localized enzymes (Lallemand et al., 2013). The interaction between these enzymes has elegantly been identified by several approaches including *in vitro* pull-down, Y2H, and FLIM/FRET assays (Lallemand et al., 2013). However, as for most other cases, the evaluation of substrate channeling is still lacking.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Given the 50 years that have elapsed since the concept of multienzyme assemblies and micro-compartmentalized metabolism (Srere, 1985) and the 40-odd years before it was postulated that these assemblies promote substrate channeling (Srere, 1987), it is perhaps somewhat surprising how little solid experimental support is available for the vast majority of the metabolons that have been proposed to operate in plants (and for that matter any other biological system). As we have detailed herein, there have been monumental advances in cell biological and biophysical approaches to identify such protein assemblies within this timeframe, as well as the advent of genomic and post-genomic tools that allow non-targeted screening of protein interactions. Indeed, a very recent study using co-fractionating MS across 13 plant species produced an amazing resource of conserved stable interactions (McWhite et al., 2020). It would be remarkable if something similar could be developed for transient interactions. Nevertheless, the advancements that have recently been made in understanding enzyme–enzyme assemblies have been phenomenal, ranging from theoretical (Sweetlove and Fernie, 2018) and mathematical evaluations (Wheeldon et al., 2016) of their function to more intricate biophysical analyses of their *in vivo* behavior. We believe that irrespective of whether they mediate substrate channeling or not, such assemblies are inherently interesting. However, we feel that a greater understanding and acceptance of their functional importance requires that the term "metabolon" be reserved for those few assemblies that have been demonstrated to channel substrates. Although such demonstrations are complicated, several routes to achieve them have been postulated, some of which are highly tractable in plants and provide very clear results. As for most areas of biology, having supporting evidence from orthogonal approaches is very important for putative PPIs, and substrate channeling is no different in this respect. With careful experimentation, we can be almost certain that many more enzyme–enzyme assemblies, and even many more metabolons, will be uncovered in the coming years, thereby further enhancing our understanding of these fascinating but enigmatic complexes.

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