

**Running Head: Transient enzyme-enzyme assemblies**

**Title: Stable and temporary enzyme complexes and metabolons  
involved in energy and redox metabolism**

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## ABSTRACT

**Significance:** Alongside well-characterized permanent multimeric enzymes and multi-enzyme complexes relatively unstable transient enzyme-enzyme assemblies, including metabolons, provide an important mechanism for the regulation of energy and redox metabolism.

**Critical Issues:** Despite the fact that enzyme-enzyme assemblies have been proposed for many decades and experimentally analyzed for at least 40 years there are very few pathways for which unequivocal evidence for the presence of metabolite channeling, the most frequently evoked reason for their formation, has been provided. Furthermore, in contrast to the stronger, permanent interactions for which a deep understanding of the subunit interface exists the mechanism(s) underlying transient enzyme-enzyme interactions remain poorly studied.

**Recent advances:** The widespread adoption of proteomic and cell biological approaches to characterize protein-protein interaction is defining an ever-increasing number of enzyme-enzyme assemblies as well as enzyme protein interactions that likely identify factors which stabilize such complexes. Moreover, the use of microfluidic technologies provided compelling support of a role for substrate-specific chemotaxis in complex assemblies.

**Future Directions:** Embracing current and developing technologies should render the delineation of metabolons from other enzyme-enzyme complexes more facile. In parallel, attempts to confirm that the findings reported in microfluidic systems are indeed representative of the cellular situation will be critical to understanding the physiological circumstances requiring and evoking dynamic changes in the levels of the various transient enzyme-enzyme assemblies of the cell.

**Keywords:** energy metabolism, metabolon, multi-enzyme complex, multimeric protein, protein-protein interaction, redox

## INTRODUCTION

Protein interactions play an important role in the proper functioning of living cells with broad-scale screens in humans have proposing the presence of approximately 130 000 binary protein-protein interactions at any point in time(1,2), thereby underlining the commonality of such interactions. Different categories of protein-protein interactions have been defined in former studies (3,4). These definitions include whether a complex is obligate or non-obligate (4). In obligate complexes the promoters of the participating proteins are not found as stable structures on their own, unlike the situation observed in non-obligate complexes. Obligate complexes mostly reflect the need for stability or the evolution of a function that requires both promoter and inter-subunit active sites, which means the protein: protein interfaces are generally larger and more hydrophobic than those associated with non-obligate associations. In addition, non-obligate complexes can exist independently as hetero-oligomeric structures such as antibody–antigen, receptor–ligand and enzyme–inhibitor complexes (3,4). Complexes can additionally be either homomeric or heteromeric in nature (4). A further distinction that is often made is that between permanent and transient complexes with the basis of this distinction being subunit affinity. Permanent protein-protein interactions are irreversible and strong, whereas the interaction qualifies as transient if it freely undergoes changes in its oligomeric state (4). These types of interactions can easily be distinguished from one another biochemically on the basis of their binding affinities which are inversely related to the dissociation constant ( $K_{off}/K_{on}$ )  $K_D$ . Whilst as illustrated in the cartoon of Figure 1 in nature these values form a continuum, permanent interactions normally display strong binding affinities (in the nM range) and weak transient interactions show rapid bound to unbound equilibrium with  $K_D$  values in the  $\mu$ M range. Such a range is in keeping with recorded local concentrations (4), underscoring the biological/ physiological relevance of such interactions. Such low  $K_D$  values do, however, render their study difficult necessitating the use of several strategies as we describe below.

In the major pathways involved in energy and redox metabolism in plants (5,6), microbes(7,8) and mammals (9), both obligate and non-obligate, homomeric and heteromeric and permanent and transient enzyme-enzyme, enzyme-protein and protein-protein interactions have been characterized (7). We will discuss these in detail in the designated sections below. However, before doing so we feel it important to provide background into (i) how these different types of interactions occur, (ii) how they are experimentally detected, (iii) how they evolved and (iv) their functional significance. Whilst we will describe all types of interactions our major focus will be on metabolons – temporary enzyme-enzyme assemblies that form between sequential enzymes of a metabolic pathway – which are involved in substrate channeling (10,11). Cellular processes have been suggested to be organized via spatial micro-compartmentalization and the assembly of multi-enzyme complexes in metabolons (12,13). The term metabolon is much overused and erroneously often simply taken to be synonymous with an enzyme-enzyme assembly as previously stated (11,14) a metabolon is a sub-class of enzyme-enzyme assemblies that acts to physically channel metabolites from the active site of one enzyme to that of another without exposure to the bulk solvent. The components of a metabolon can be specific to a single metabolon or dynamically shared between metabolons for swift adaptation of metabolism to environmental challenges and cellular needs (11,13,14). Such an organization of metabolic pathways at the molecular level has been postulated to have several advantages including (i) increasing local concentrations of the enzymes and their substrates, (ii) improving channeling of intermediates into specific sub-pathway, (iii) increasing metabolic fluxes and (iv) sequestration of reactive intermediates (15-17). However, the evidence in support of these postulates varies depending on the metabolon in question and as we have previously stated (14). Indeed, a more likely universal reason for their formation is to ensure that metabolic fluxes are regulated in a manner befitting the cellular circumstance. Before discussing both stable and transient complexes in specific pathways we felt it useful to provide a brief historical overview of their study. Our main focus will be on enzymes, however, we will also mention both members of electron transport chains and transporter proteins where these have a strong influence on metabolism. Throughout the manuscript we adopt the terms enzyme-enzyme assembly for enzymes that are merely demonstrated to interact reserved the term and metabolon for

the subset of enzyme-enzyme assemblies for which substantial evidence of metabolite or substrate channeling exists. We intend to provide case study analyses of five key pathways of energy and redox metabolism namely, glycolysis, the tricarboxylic acid cycle (TCA cycle), the mitochondrial electron transport chain, the Calvin-Benson cycle and photorespiration before attempting to define general features of the enzyme assemblies in these pathways and to interpret why at certain steps of the pathway stable as opposed to transient interactions have evolved and *vice versa*. Finally, we review exciting pertinent developments emanating from cell free systems. Whilst stressing that proof for their physiological relevance is currently lacking, we expound on the potential importance of these findings. Wherever possible we will review the available data at a cross-kingdom level.

## TYPES OF ENZYME-ENZYME INTERACTIONS

### MULTIMERIC ENZYME COMPLEXES

There are a staggering number of enzymes which only function or only optimally function as multimeric enzyme complex characterized by permanent interactions, examples of such enzymes are aldolases, oxidases, catalases, many galactosidases and many dehydrogenases (11,18). With regard to the pathways this review is concerned with a complete list of multimeric enzyme complexes is provided in Table 1. In cases where proteins are multimeric, attachment of one enzyme to several scaffold proteins could allow cross-linking to form larger structural protein complexes, which often possess low stability (19,20). In addition, the stabilization of multimeric proteins relates to the dissociation of the enzyme subunits or the loss of their correct assembly structure (18). Thus examining multimeric enzyme complexes depends on experimental conditions which mimic the enzymes native environment (presence of ions, polymers, etc.), the existence of inter subunit disulfide bridges, or chemical- or physical- crosslinking with poly-ionic polymers (18). Given that there is a vast literature concerning the study of such multimeric enzyme complexes (18-21) and the fact that they appear to be universally characterized by co-evolution of the many residues which mediate their binding at the subunit interface (18), we will not extensively discuss such interactions here with the

exception of a brief comparative evaluation of the evolution of permanent and transient enzyme complexes in *THE EVOLUTION OF PROTEIN COMPLEXES* below.

## MULTI-ENZYME COMPLEXES

Several copies of one or several enzymes (polypeptide chains) are stably associated by strong permanent non-covalent interactions in multi-enzyme complexes, which carry out a single or a series of biochemical reactions such as the pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, glycine cleavage system, fatty acid synthase, glutamine synthase, proteasome and Rubisco complexes (22). As above, with regard to the pathways this review is concerned with a complete list of multi-enzyme complexes is provided in Table 1. These multi-enzyme complexes have been studied to varying levels and whilst for some the subunits are unique to the complex for others the subunits are shared between complexes for example mitochondrial dihydrolipoyl dehydrogenase (mtLPD) is shared as an integral component of several multienzyme systems including the tricarboxylic acid cycle (pyruvate dehydrogenase complex, PDH, 2-oxoglutarate dehydrogenase, OGDH), photorespiration (glycine decarboxylase complex, GDC), and the degradation of branched-chain  $\alpha$ -ketoacids (branched-chain 2-oxoacid dehydrogenase complex, BCDHC)(23,24). The reaction intermediates of multi-enzyme complex are entirely enclosed in internal chambers within the protein complex, which renders directly diffusion between active sites of the component enzymes or use swing arms to physically mobilize intermediates or cofactors (14). Multi-enzyme complexes can dramatically improve the catalytic efficiency of the reaction chains in question having even been argued to have reached catalytic perfection (25,26). Given that channeling clearly exists in these complexes, their comparison with metabolons is particular apt and we also attempt this in *THE EVOLUTION OF PROTEIN COMPLEXES* below. Like the multimeric enzymes described above the mechanism underlying the interaction are largely understood, although the presence of subunits in multiple different protein complexes is probably more common for this type of interaction rendering the evolutionary pressure that, at least some of, the subunits receive unequal. Unfortunately, due to space constraints, we cannot comprehensively review this type of permanent enzyme complex here so rather refer the reviewer to a number of excellent recent reviews on this subject (19,27-29).

## TRANSIENT ENZYME-ENZYME ASSEMBLIES

Transient enzyme interactions include protein interactions which are formed and broken easily i.e. relatively unstable complexes, which play an important role in many aspects of cellular function (4,7,30). Transient interactions are characterized by dissociation constants ( $k_{\text{off}}/k_{\text{on}}$ ) -  $K_D$  - in the micromolar range and lifetimes of seconds (Figure 1), while strong transient interactions may have lower  $K_D$ 's in the nanomolar range and longer lifetimes. Commonly weak transient complexes are represented by dynamic mixtures such as protein phosphorylation or dephosphorylation in vivo, whereas strong transient complexes change their quaternary state for example when triggered to do so on ligand. Although transient protein interactions play an important role in biological systems, notably in regulating the dynamics of biological networks, their experimental detection is not easy (30). In addition, the transient interactions of sequential enzymes of several metabolic pathways may well participate in the substrate channeling formation inside living cells. As such metabolons represent a subset transient assemblies, relying on delicate local changes in solutes, structural elements and possibly scaffolding proteins to allow their operation (15). Given the importance of this subset of assemblies we will discuss them in detail below.

## THE METABOLON

The concept of physical enzyme-enzyme complexes was initially conceived in 1970 by A. M. Kuzin of the USSR Academy of Sciences (31), and subsequently adopted in 1972 by Paul A. Srere of the University of Texas for the enzymes of the citric acid cycle(32). The hypothesis that structural assembly of enzyme was common and explained many of the inconsistencies between theoretical expectations and biological observations was well accepted in the former USSR and further developed in by B.I. Kurganov and A.E. Lyubarev's finding of complexes of glycolytic enzymes (33,34). In the mid-1970s, the group of F.M. Clarke at the University of Queensland, Australia also provided considerable support for the concept (35). The name "metabolon" was firstly coined in 1985 by Paul Srere during a lecture in Debrecen, Hungary(36), who went on to provide the first detailed experimental study of the phenomenon while working on the mitochondrial tricarboxylic acid cycle (10,37). From these studies he notes that "through the dynamic association and/ or

dissociation, transient complexes allow the regulation of metabolic pathway flux (11,12,38). As mentioned above metabolons mediate “substrate channeling” (also known as metabolic channeling) wherein reaction intermediates are isolated from the bulk environment surrounding them. Various advantages of substrate channeling have been postulated including isolation of intermediates from competing reactions, local enrichment of metabolite concentrations in order to achieve high reaction rates, sequestration of cytotoxic metabolites and protection of unstable intermediates (37,39,40). Furthermore, during the functioning of metabolons, the amount of water needed to hydrate the enzymes is decreased and enzyme activity is enhanced. A vast number of metabolons have been proposed to mediate substrate channeling in various organisms; including those proposed to operate in branched chain amino acid metabolism in human mitochondria (41), the glycolytic pathways of mammals, yeast and plants (42-44) and a wide variety of specialized metabolic pathways including polyamine (45), isoprenoid (46), alkaloid (47) and a number of different phenylpropanoid pathways (including. lignin, carotenoid, flavonoid, isoflavonoid and cyanogenic glucoside synthesis in plants (16,48-51). That said, strictly speaking, metabolite channeling must be observed in order to claim the presence of a metabolon and not all enzyme-enzyme assemblies are metabolons. Based on this requirement there is relatively limited evidence for the presence of metabolons. Indeed to our knowledge in plants: only glycolysis (42), the TCA cycle (10), and the cyanogenic glucoside biosynthetic pathway (16) have been demonstrated to operate in this way with all other structures that were described as metabolons to date being more accurately described, on the basis of cumulative evidence, merely to be enzyme-enzyme assemblies. This is not said to diminish the importance of the interactions in question, merely to reinforce a more rigorous definition of the term metabolon (11,14).

## **EXPERIMENTAL EVALUATION OF ENZYME-ENZYME INTERACTIONS AND SUBSTRATE CHANNELING**

We have discussed above how protein-protein interactions could drive both metabolic and regulatory events and how large macromolecular complexes are highly stable and even permanent, whilst dynamic and transient interactions are key components with regard to signaling, regulatory networks and the formation of substrate channels (4,30).



Given the instability of transient complex formation, it is technically difficult to study and harder to detect weak protein-protein interactions, compared with the more stable interactions exhibited by multimeric enzymes and multi-enzyme complexes. That said high-throughput yeast two-hybrid (Y2H) screens are able to detect binary transient interactions (4). However, similar to other experimental detection methods of protein-protein interaction, Y2H has its own weaknesses, including a high rate of false-positive detections with attempts to ameliorate this problem often resulting in a reduced overall ability of the method to detect transient interactions (52). Despite this fact, it is still the popular method for large-scale detection of protein-protein interactions, owing to its scalability and accessibility (52). Furthermore, several recent developments to this approach enabled the detection of additional types of interactions including those involving cytosolic, extracellular, or membrane-bound proteins (16,53). These developments notwithstanding proof of complex formation are now almost universally required to not be based on a single technique alone and as such Y2H is normally used in tandem with another technique or in teaming with multiple techniques. In keeping with this development several research efforts have focused on bioinformatical integrating different protein-protein interaction methods in order to gain reliable interaction lists (54). For instance, the recent database STRING V11, collects, scores and integrates all publicly available experimental sources of protein-protein interaction information, and allows comparison of these with computational predictions (54). In addition to this, considerable research works has focused on decreased the problem of false negative protein-protein interactions by improving the protein interaction method. For example, two recently developed approaches namely bioluminescence resonance energy transfer (BRET) (55) and fluorescence/Foerster resonance energy transfer (FRET) (56) 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 and mCherry. After introducing the fusion proteins via transgenes, BRET and FRET between the two proteins are measured (55,56). These approaches were demonstrated to have the additional benefit of highlighting transient, membrane-bound and extracellular protein interactions, since they detects the interactions on the basis of physical distance (30). Measurements yielded by these methods thereby supply a non-invasive means by which to visualize the

spatiotemporal dynamics of interactions between protein partners *in vivo*. In addition, use of confocal laser scanning microscopy (CLSM), may enable the direct imaging of substrate channels following the transient or stable expression of target proteins as immunologically tagged proteins or as fluorescent-fusion proteins (15,40). Direct visualization of substrate channel, allowing assessment of their association by sequential incorporation of their component polypeptides, can now also be achieved by atomic force microscopy or the fluorescence lifetime imaging microscopy based FRET (FLIM-FRET) (16). Moreover, new methodologies such as the use of Clear-Native-PAGE, cross-linked mass spectroscopy, proximity dependent biotin identification (BioID) and ascorbic acid peroxidase (APEX) enable the isolation and characterization of enzyme-enzyme assemblies in which adherence between the different subunits is weak (57-59). Another well-developed high-throughput protein-protein interaction detection method is affinity purification followed with mass spectroscopy (AP-MS) (60,61), which allows rapid purification protein complex under native conditions, even when expressed at their native level. As several washing steps are required in order to remove nonspecific binding, this method has traditionally been unable to detect the transient interaction. However, via the use of *in vivo* chemical crosslinking, it has become possible to detect transiently formed complexes by inducing covalent-bond formation between interacting proteins (62). Additionally technologies subsequently allow the preservation of the crosslink during the washing phase of affinity purification (62,63).

Despite advances in the implementation of the above-described techniques a functionally important subset of transient interactions is dependent on post-translation modification events. Such interactions are often missed when protein-protein interaction screening is performed in yeast two hybrid (62). This limitation has been tackled in different ways, including the use of native cell culture systems, an approach that remains constrained by the difficulty of transforming either mammalian or plant cell cultures. Among other methods that feature the use of endogenous cell systems for protein-protein interaction screening are bimolecular fluorescence complementation (BiFC) (61,64,65) and the split-luciferase assays (66) which are both able to detect transient (as well as permanent) protein-protein interactions *in vivo* thereby eliminating the need for high-throughput

purification studies. The characteristics of the various experimental techniques to identify transient protein-protein interactions are summarized in Figure 2.

Recently developed approaches give us various options to test protein–protein interactions (64,67). Indeed, the reports discussed here utilize various techniques to investigate protein complex formation as well as the interactions between specific enzyme pairs (Figure. 2). However, there is still no perfect approach which can study protein–protein interactions without any false negative and positive detection. This renders the validation and screening of novel enzyme-enzyme assemblies complicated. Yeast two hybrid assays, co-localization of fluorescently tagged proteins, BiFC, and co-purification are commonly used to identify enzyme-enzyme complexes (14,60,64,65,67). All these approaches tend to generate a lot of false positive results and have to be conducted with special care, paying particular regard to the use of proper negative controls (64,65). Additionally, given the relatively high false positive and negative rates of these assays, evidence from multiple independent approaches are required in order to accurately call the presence of enzyme and enzyme assemblies (10). 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 research comes to the screening of protein complexes within a large set of enzymes or interacting protein pairs, it becomes increasingly complicated to gain reliable results. The use of multiple techniques is, therefore, 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 the enzymes find themselves in. As such use of a single approach may provide an improper microenvironment resulting in false-negative results. Conversely problems arise to integrate the 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. In the screening of protein–protein interactions within the plant TCA cycle, combination of three rather conventional methods, split luciferase complementation, yeast two hybrid, and AP-MS was used (10,68). All three of these approaches provide information on in vivo protein-

protein interactions yet the principle of detection in all instances differs from one another. Importantly, however, they generate semi-quantitative scores which can be statistically combined to produce a single reliability score. This method successfully identified expected and novel elements of plant TCA cycle metabolon (69) and as such provides a robust framework for the screening of physiologically occurring enzyme-enzyme assemblies.

Whilst the above techniques represent the state-of-the-art in detecting enzyme-enzyme assemblies the specific identification of metabolons requires stricter proof than is commonly provided (10,11,14). Identification of enzyme-enzyme assemblies that include consecutive enzymes certainly represent good candidates for complexes which may contain metabolons. That said before describing such assemblies as metabolons or even metabolon containing proof of substrate channeling is required. As mentioned above this has been achieved for a subset of the reactions comprising the glycolytic and TCA cycle pathways (10,42,63,70). Such proof is non-trivial. Given that it is debated in length in a recent review (14) we will not re-iterate the arguments made there merely to briefly state that both isotope labelling (14,42,71) and non-isotope labelling approaches (19,72,73) have been used with those of greatest relevance to the pathways covered in this review being summarized in Figure 3 and 4.

## THE EVOLUTION OF PROTEIN COMPLEXES

A relatively recently expanding research field concerned with enzyme interactions concerns the evolutionary histories of stable enzyme multimers, multi-enzyme complexes and dynamic assemblies of enzymes (10,14). In this vein considerably more evolutionary based research has been implemented on the former—with many permanent interactions being highly conserved throughout evolution. For example, pioneering research proposed that the  $\alpha$ -ketoacid dehydrogenase complexes appeared very early in evolution, being found in aerobic members of the bacteria, archaea and eukaryotes(74). Another well-studied multi-enzyme complex is the fatty acid synthase complex (FAS)—of which two amazingly different types have evolved in eukaryotes: the fungal and metazoan FAS (75). the fungal FAS associates a 2.6-MDa assembly comprising 48 functional domains and,

alongside the homologous CMN-FAS recently described in *Corynebacterium*, *Mycobacterium* and *Nocardia*(76), constitutes one of the most complex biochemical protein machineries uncovered to date (77), while the metazoan FAS is a 540-KDa homodimer that shares a common architecture with bacterial polyketide synthases(78). A recent study determinate *trans*-acting polyketide enoyl reductases and non-canonical bacterial fatty acid synthases as potential ancestors of the scaffolding regions with a striking conservation of insertions to scaffolding components despite minimal sequence identity (75). In contrast, many bacteria and plants harbor only monofunctional FASs (79). Another classical example of a multi-enzyme complex is the penta-functional Arom complex of shikimate biosynthesis which appears likely to have evolved via gene fusion with the enzymes of most other species being monofunctional (80). A final multi-enzyme complex that merits discussion is that of tryptophan synthase (81) for which a plausible model by which the sophisticated multi-enzyme complex evolved via stepwise association of protein subunits has been proposed (81,82). Research into the evolution of protein complexes is currently largely focused on residues at the interface of the subunits and as such on their co-evolution (83,84).

The evolution of metabolons (and indeed of other transient enzyme-enzyme assemblies), is far less studied. However, as presented in Table 1, these demonstrate a similar range of conservation throughout evolution as the stable multi-enzyme complexes. In particular, metabolons within glycolysis and the TCA cycle are highly conserved. Others, such as the urea/polyamine substrate channel, are found, at least in part, in several species. Yet, other substrate channels, especially those involved in fungal and plant specialized metabolism, are however far more limited in their species range. That said, despite the widespread occurrence of metabolons (and other types of 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 (14). What is almost certain, however, is that less conservation at the interface of the interacting proteins will be apparent in metabolons and other transient enzyme-enzyme assemblies. Moreover, in philosophizing about the role of these transient structures one

could conceivably argue that the flexibility afforded by their transient nature is likely to provide an advantage, rather than a compromise, to fitness. Since this clearly was not the case for multimeric enzymes and multi-enzyme complexes begs the fascinating question as to what properties render a certain reaction in an enzyme cascade, or alternatively the protein that catalyzes it, a good candidate to stabilize and which characteristics rather lead to the maintenance of (relative) plasticity?

We will return to the above questions later. On a more general note the presence of protein aggregates has been argued to be under negative selection (10). However, at the same time protein aggregation may be an unavoidable consequence of the limited solvency capacity of the cell in relation to the total protein concentration rendering as such functional organization around such aggregates an evolutionary compromise (85). Given that a wider range oligomeric proteins can adopt multiple structural conformations, there may be unique opportunities for mutation to lead to novel protein–protein contact points (10). While in many cases novel aggregations will be deleterious, some will be neutral and may even ultimately prove beneficial to the cell (85), in which case it would seem reasonable to anticipate their stabilization. However, recent studies of protein interaction data in the context of evolution have left several unanswered questions (86). Most important 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 and (iii) are mutations in the binding site of an interacting protein correlated to those in the binding site of its partner (10). Fascinatingly, studies aimed at answering these questions observed that amino acid residues at the interfaces of obligate complexes evolved at a relatively slower rate allowing them to co-evolve with their interacting partners (83,84,87). By contrast, when the protein–protein interactions are transient, the rate of residue substitution is considerably higher (87). These observations are thus in keeping with our suggestion that multi-enzyme complexes and metabolons evolved independently from one another. As such these result in distinct repurposing of the ancestral enzymes. We have previously proposed that the key functional discriminator is the requirement for access of competing enzymes to the channeled metabolite so that metabolic branch points can operate. This would require

disassembly of the enzyme complex that supports substrate channeling and would provide a selective pressure against the evolution of a stable multi-enzyme complex for such a set of reactions (10). Put more simply, we believe that stable multi-enzyme complexes can only evolve where there are no other cellular reactions that requires access to the channeled metabolite, in the absence of such conditions there is no selective advantage in conservation of residues that improve interface binding and as such the complex process of co-evolution is unnecessary (10). To date very few studies have been carried out looking at the evolution of metabolons but following the assumptions we state above we would anticipate much higher conservations of the residues involved in hollow-core or electrostatic channeling of metabolites than at the protein-protein interaction interface (88). The results for enzyme-enzyme interactions which do not lead to substrate channeling are, however, much harder to predict.

## **PROTEIN COMPLEXES IN RESPIRATORY AND PHOTOSYNTHETIC METABOLISM**

### **RESPIRATORY METABOLISM**

Respiratory metabolism including the pathways of glycolysis, the TCA cycle and the mitochondrial electron transport chain provides carbon skeletons for biosynthesis of a series of key metabolites and as such is a central feature of metabolic networks across all kingdoms of life and the core of biochemical energy transformation (11). The basic electrochemistry and chemistry of the pathways are highly conserved (89,90) with the exception of energy parasites such as chlamidiae, mistletoe and diatoms which have reduced respiratory function (91-94). Despite the fact that the pathways operate in widely different physiological settings, a degree of metabolite channeling - the promoted transfer of the metabolite from one enzyme to the sequential enzyme in the metabolic pathway without that intermediates equilibrating with the large amount aqueous solvent (14) - appears to be a common feature of these pathways across all kingdoms of life. Here we will direct the detail of this aspect in respiratory metabolism, describing the nature of metabolite channeling for three metabolic pathways. However, we will additionally detail other enzyme-enzyme interactions i.e. those that do not, or at least have not yet been demonstrated to, result in channeling. Furthermore, we will discuss these transient

interactions alongside discussion of the cardinal multi-enzyme (and multi-protein) complexes that operate within these pathways.

## GLYCOLYSIS

The observation of transient assemblies of glycolytic enzymes has an extensive history. Initial, albeit controversial and indirect evidence, came from the evidences of independent pools of glycolytic metabolites in rats and *E. coli* (95,96). Similar evidence of sub-compartmentation of metabolite within the cytosol has subsequently been provided by high resolution non-aqueous fractionation in the model plant *Arabidopsis thaliana* (97). However, considerably stronger evidence for assemblies of glycolytic enzymes has been provided such as direct proof of specific protein-protein interactions between constituent pairs of glycolytic enzymes (11,14), association of enzymes of glycolysis with actin (98,99) or red blood cell membranes(96,98),and complexes of all glycolytic enzymes (96,98). There is an ample body of research that glycolytic enzymes associated with red blood cell membranes (100) with the environmentally dependent binding affinity (101). Several glycolytic enzymes (glyceraldehyde phosphate dehydrogenase, aldolase, pyruvate kinase and lactate dehydrogenase) were proved to bind to an immobilized F-actin-tropomyosin complex (102). However, as a recurrently observed pairing, the specific interaction between constituent enzymes of aldolase and glyceraldehyde phosphate dehydrogenase is the real breakthrough in building the occurrence of glycolytic enzyme associations(103). These interactions were revealed by a diverse range experimental approaches including polarization of fluorescence, kinetic studies, affinity electrophoresis (104) and active enzyme centrifugation. However, intriguingly a simple gel filtration of cell extracts of *S. cerevisiae* (103) and *E. coli* (105) revealed only a small proportion of the enzymes to be in complex with one another. There is additionally a large body of evidence which proposes interaction of glycolytic enzymes with mitochondria. For example, a study of *Tetrahymena pyriformis* reported that 100% of lactate dehydrogenase, 75% of phosphofructokinase and 50% of glyceraldehyde phosphate dehydrogenase are associated to mitochondria (100-102), whilst association of hexokinase to the mitochondrial outer membrane was believed early to be a general character of eukaryotes (103). It has been postulated that a mitochondrial location of hexokinase was beneficial in terms of energy efficiency,



providing ready access to the ATP being generated by the mitochondria leading to the suggestion that metabolic channeling occurs, with associated kinetic and regulatory benefits (103) (Figure 5). We have previously questioned this interpretation (11), however, this fact notwithstanding, these studies nicely provide quantification of the amount of assembled as opposed to non-assembled activities – something that unfortunately is rarely provided.

The above-described evidence for glycolytic complex formation was considerably boosted by a wealth of cross-kingdom evidence this century (14,106). A handful of proteomics studies have suggested the presence of the enzymes of glycolysis in isolated mitochondrial fractions from *Arabidopsis* (70), humans (107) and yeast (43). Studies employing both blue native SDS-PAGE and enzyme assays as well as co-immunoprecipitation of proteins with anti-enolase antibodies demonstrated that enolase is a member of a large macromolecular protein complex assembled to the mitochondria and also including enzymes of the TCA cycle and mitochondrial membrane carriers in yeast (108). In *Arabidopsis*, protease treatments were used to demonstrate that these enzymes were mainly localized on the cytosolic face of the outer mitochondrial membrane (70). Subsequent experiments in which the glycolytic substrates  $^{13}\text{C}$ -glucose and 1- $^{13}\text{C}$  fructose-1, 6-bisphosphate were supplied to isolated mitochondria demonstrated that the complete glycolytic sequence was present and active in this fraction. Further research - this time working on potato mitochondria – showed that this association was dynamic in that inhibition of respiration by KCN led to a proportional decrease in the association of glycolytic enzymes with mitochondria whereas conversely stimulation of respiration enhanced this association. Detailed study suggested that this association is mediated by VDAC which anchors the glycolytic enzymes to the membrane and is the outer mitochondrial membrane protein and (42). Importantly, this research also provided indirect evidence for the channeling of the glycolytic metabolites with the labelling patterns of glycolytic metabolites being followed by NMR proposing a leaky channeling of glucose 6-phosphate and fructose 6-phosphate but a tighter channeling of intermediates from fructose 1,6-bisphosphate onwards (42). Channeling, albeit to a lesser extent, has also been demonstrated for

mammalian glycolysis (109), and even in species, such as *E. coli*, which lack mitochondria (110).

Two approaches have proven particularly informative in assessing the presence of metabolite channeling in glycolysis namely analysis of modelling of  $^{13}\text{C}$  isotopic labelling studies and NADH channeling (Figure 4). The first of these was an early experiment by Srivastava and Bernhard which demonstrated the direct transfer of NADH from liver glyceraldehyde phosphate dehydrogenase to alcohol dehydrogenase (111). Further work from this lab has demonstrated that a series of such transfers can occur between dehydrogenases and that in every case the transfer occurs between dehydrogenases with alternate stereospecificity for NADH. The second method was additionally able to indicate that modelling data acquired from isotope labelling assays in a pattern which consists of channeled glycolysis provides a better fit to the result than when channeling of this pathway is not considered (25,112). There are six further recent publications regarding glycolytic assemblies – a review on the activity of glycolytic metabolons in muscle (113), three cell biology studies on glycolytic assemblies in animals (26,44,114), a study of the glycolysis actin interaction in yeast (43), and finally an example of chemotactic driven enzyme assembly using glycolytic enzymes and intermediates in a cell-free system (115). Given that we reviewed these recently (11) we will only detail the last of these here. This study reported the fascinating recent finding that using microfluidic and fluorescent spectroscopic techniques it was possible to demonstrate that the first four sequential enzymes of the glycolytic pathway each independently abides by its own specific substrate gradient. As such this study provided considerable insight into one potential mechanism for the assembly of transient enzyme complexes (115). Indeed, this finding opens up an additionally possible physical route, alongside the above-described cytoskeletal and membrane platforms, by which enzyme-enzyme interactions can nucleate. Indeed, there is an ever-increasing body of evidence, as we detail in *STRUCTURAL SUPPORT AND NUCLEATION POINTS FOR ENZYME-ENZYME ASSEMBLIES* below, that this mechanism is one that can be demonstrated, at least *in vitro*, to occur in a range of pathways.

## THE TCA CYCLE

The TCA cycle is one of the iconic pathways in metabolism being elucidated by Hans Krebs in 1940 (116), although some cross-kingdom variation in this pathway has been observed (117), the recent elucidation of an alternative route from 2-oxoglutarate to succinyl-coenzyme A (118) means that these differences are more subtle than initially thought. Indeed, several different bypaths of the TCA cycle exist ranging from the nearly universal GABA shunt to more taxonomically restricted bypasses such as malate and acetate shunts (117). Aside, from these minor differences, the TCA cycle is, however, remarkably conserved.

As for glycolysis, the search for dynamic enzyme assemblies in the TCA cycle was largely driven by the need to explain kinetic observations that did not fit the conventionally accepted view of a well-mixed metabolism (103). Srere et al. further proved that an immobilized pairing of malate dehydrogenase and citrate synthase had a kinetic benefit over the diffusion enzymes (119). A variety of other early experiments using a wide range of diverse approaches provided further support for the organization of the TCA cycle enzymes that Srere first termed the metabolon (11,37)). Since these pioneering studies, dynamic aggregation of consecutive enzymes of glycolysis have been observed across the kingdoms of life (11).

Over a period of twenty-five years studies, either by or inspired by Srere and Sugemi and their co-workers, applying gel filtration and precipitation in polyethylene glycol discovered physical protein interactions between six of the eight enzymes of the pathway (11). The importance of these researchers cannot be underestimated since their research cannons additionally include the first structural model of the malate-dehydrogenase-citrate synthase-aconitase complex (38) and the determination of dissociation contents of the complex following the incubation of a fluorescein isothiocyanate labelled citrate synthase with malate dehydrogenase in pig heart and a series of intermediates intimately related to the TCA cycle. Interestingly, 2-oxoglutarate increased the dissociation constant whilst NADH lowered it. The effect of 2-oxoglutarate remains difficult to understand, however NADH is a major determinant of the energy-generating flux of the mitochondria (11)

Again, as demonstrated for glycolysis above, the advent of fluorescence-based cell biology and proteomics facilitated the study of pathway wide interactions of the TCA cycle. For example, a comprehensive characterization of the interactions between *Bacillus subtilis* enzymes revealed interactions between six sequential enzymes of the TCA cycle. Moreover, the TCA cycle enzyme complexes' structural models are consistent with metabolite channeling via electrostatic retention of the channeled metabolite on charged domains of the enzyme surfaces (63,120). Similarly, as already described above a comprehensive range of techniques including affinity purification mass spectrometry, yeast two-hybrid assays and split-luciferase have been used to demonstrate protein-protein interactions in the plant TCA cycle (10). One surprising feature of wide-scale protein interaction studies is the substantial number of interactions between non-consecutive pathway enzymes. It is conceivable that many of these interactions, which occur largely between regulatory subunits of one enzyme and the catalytic subunit of another, act as nucleation points which aid in the formation of metabolons (10) (Figure 6). In a recent study the extra-pathway interactions of the plant TCA cycle enzymes were revealed, with 125 interactions being identified that highlighted many novel interactions, including the amino acid metabolism, mitochondrial electron transport chain complexes (mETC), ATP synthesis, signaling, lipid metabolism, nitrogen metabolism and redox stress(68) (Figure 6). Interestingly, the ATP synthase interacted with the succinate dehydrogenase (121), and proposed to organize the super-complex with TCA cycle metabolon in the inner membrane of mitochondria (122). In addition, several subunits of succinate dehydrogenase were found to interact with the other of other complexes of the mETC. These results also support the postulated super-complex of the mitochondrial electron transfer chain complex(123).

In a more recent study Wu and Minter (63) coupled *in vivo* cross-linking and mass spectrometry to build a low resolution model of the malate dehydrogenase-citrate synthase- aconitase complex. Interestingly, this revealed protein interactions between all the TCA cycle enzymes. Employing distance constraints derived from the crosslinking, two possible structure models of the malate dehydrogenase-citrate synthase- aconitase protein complex were suggested. The average distances of aconitase-citrate synthase and

malate dehydrogenase-citrate synthase between the active site are, 50 Angstrom and 35 Angstrom respectively, in the model based on the most prevalent protein structure.

A theoretical research by Elcock and McCammon (124) suggested that the existence of electrostatic interactions could highly increase substrate-transport efficiency. Moreover, recent research, from the same lab, suggests that, as demonstrated for glycolytic enzymes above, metabolite levels gradients increase association of the TCA cycle metabolon (63).

As recently reviewed (11), a variety of isotope labelling methods have also been used to provide evidence for metabolic channeling in the TCA cycle. Arguably more importantly labelling methods have also been employed as a more common test of the existence of channeling. For instance, the 'isotope dilution' approach uses the range of dilution of labelled intermediates pools following addition of amounts of unlabeled metabolites as a measure of metabolite channeling. For instance, Zhang et al., fed purified mitochondria of potato tuber with  $^{13}\text{C}$  labelled glutamate or pyruvate until accumulation of  $^{13}\text{C}$  in the following TCA cycle intermediates achieved isotopic steady state (10). Then unlabeled TCA cycle intermediates were supplied and the "dilution" effect on labelling was followed over time. In order to avoid the complications in interpretation of labelling patterns caused by multiple turns of the cycle, the TCA cycle was linearized by inhibiting succinate dehydrogenase with malonate in the pyruvate feeding assay and by inhibiting aconitase with fluoroacetate in the glutamate assay. These experiments exposed dilution in 2-oxoglutarate, malate and succinate but none in fumarate or citrate indicating that the latter metabolites are channeled. Intriguingly, the data from a metabolic flux investigation due to  $^{13}\text{C}$ -label redistribution in heterotrophic *Arabidopsis* cell culture suggested channeled flux from fumarate to malate but no channeled flux from 2OG or succinate to citrate (125). More detailed studies in potato tuber mitochondria following  $^{13}\text{C}$ -labelling demonstrated channeling of both fumarate and citrate(10).

An alternative non-structural approach that warrants discussion is the so-called enzyme buffering approach which has been extensively used in studies of NADH channeling by NADH dehydrogenases. In this method the question addressed is whether the second enzyme (E2) uses the enzyme 1 (E1) bound form of the common intermediate as well the

free form. Given that the dissociation constant  $K_D$  of E1-NADH is approximately 1 mM in the presence of excess E1 it is possible to decrease  $[NADH]_r$  to a value well under its  $K_m$  for E2. The major arrow in Figure 4 suggests that for such a binding equilibrium NADH is more than 99% in its E1 bound form. Therefore, if the experimental tested reaction rate is highly in excess of that which could be reached from free NADH alone then this provides evidences that the NADH is channeled between the sequential enzymes (112). Intriguingly, NADH channeling has only been found in consecutive enzymes of opposite chirality but clear cut results have been reported for several such enzyme pairs (112). By contrast, if NADH is not channeled then E1 is simply buffering NADH to a low  $[NADH]_f$  (39). Whilst currently only reported for NADH containing reactions, this method still has broad utility given the high number of dehydrogenase reactions in the cell and their importance in energy and redox metabolism.

## THE MITOCHONDRIAL ELECTRON TRANSPORT CHAIN

The mitochondrial electron transport chain, is more divergent than the other respiratory pathways with considerable divergences in alternative respiratory pathways and the sizes of the respiratory complexes being proposed in plants (126). It has, however, been studied for at least as long as glycolysis and the TCA cycle (127). More recently, our understanding of the process has been advanced by molecular-level information concerning the structures of the protein complex and electron-transport complexes that catalyze oxidative phosphorylation and constitute the respiratory chain (127,128). These studies resulted in our current understanding of the three proton translocating complexes, complex I, III and IV as well as the mobile electron carriers cytochrome c and ubiquinone (11,128), with the mechanism underlying the proton-motive force required for ATP synthesis now being very well investigated (Figure 7).

The respiratory complexes are well-determined stable multi-subunit protein complexes that have been subjected to substantial research programs given the significance of respiratory chain dysfunction in disease and aging (127). Indeed the separate respiratory protein complexes could be dynamically assembled into super-complexes as observed in plants (129,130), mammals (131-133), yeast (131); and even some bacteria such as

*Paracoccus denitrificans* (11,134). However, some species-specific variations between these super-complexes have been observed (Figure 7). For instance, patches of identical complexes is suggested in different parts of its cell membrane in *E. coli* (135), while BN-PAGE has proved super-complexes of varying stoichiometries with complexes I and III being far more similar to be discovered in a super-complex than complex IV (132). Additionally, only simpler super-assemblies of complexes III and IV were observed in *S. cerevisiae*, which does not contain complex I, (136,137). That said, despite initial skepticism, respiratory super-complexes are now firmly accepted (11). However, the question whether they confer any function advantage and the reasons for their existence remain much debated (127). Indeed, by contrast to the glycolytic TCA cycle pathways, evidence that the super-complexes perform metabolite channeling remains considerably contended with researchers debating whether structural proof is consistent with the presentence of substrate channeling or not (123,138-140). If this conclusion is correct the question of the physiological purpose of super-complex formation becomes key? Alternative suggestions including (i) modulation of cristae morphology regulation of respiratory chain activity, (ii) stabilization of the individual complexes, and (iii) the prevention of protein aggregation in the highly crowded mitochondrial membrane (127). That said considerable further research is required in order to assess the relative likelihood of these hypotheses.

## PHOTOSYNTHESIS

Unlike heterotrophic systems, photosynthetic tissue capture light energy (141). In the two steps of photosynthesis, light-dependent and light-independent (i.e. the Calvin–Benson cycle) reactions depends on several enzyme complexes. In addition the process of photorespiration – a metabolic repair cycle – which returns photosynthesis to the Calvin–Benson cycle following the oxygenation, as opposed to carboxylation, of ribulose biphosphate(142). Despite the fact that the pathways functions in widely different physiological settings, the emergence of new kinetic and regulatory properties as a consequence of protein–protein interactions will be discussed in order to aid understanding into how flux is regulated through these pathways. Our discussion of these pathways will be shorter than the previous ones not only because they are taxonomically

narrower but also given that less evidence is currently available concerning enzyme-enzyme assemblies in this pathway.

### 1. The Calvin-Benson cycle

In the micro-compartment of the chloroplast stroma, the enzymes involved in the Calvin-Benson cycle pathway are not randomly distributed, but interact to the multi-enzyme complex (141). For example, a potential multi-enzyme complex which comprised five pathway enzymes, ribose-phosphate isomerase (RPI), phosphoribulokinase (PRK), ribulose-bisphosphate carboxylase/oxygenase (RuBPCase), phosphoglycerate kinase (PGK), and glyceraldehyde-phosphate dehydrogenase (GAPDH) has been proposed (141,143,144). In the further experiment, the 900Kd stable super-complex of Calvin-Benson cycle was organized by enzymes RPI, ribulose-5-phosphate kinase (Ru-5-P-K), RuBPCase, GAPDH, sedoheptulose-1,7-bisphosphatase (Sed-1,7-bPase) and the electron transport protein ferredoxin-NADP<sup>+</sup> reductase (FNR) (143). Immunoelectron microscopy revealed that Ru-5-P-K, GAPDH, Sed-1,7-bPase, and FNR are bound to stroma-faced thylakoid membranes *in situ*, whereas rubisco and rubisco activase are randomly distributed throughout chloroplasts. The interaction between GAPDH and aldolase was demonstrated by several method in the pea chloroplast (*Pisum sativum* L.) (145), whilst multi-enzyme complexes of varying compositions of Calvin-Benson cycle enzymes have been isolated from pea and spinach most predominant being the PRK/GAPDH complex (144,146,147). This complex may, therefore, correspond to the core complex of a super-complex involved in CO<sub>2</sub> assimilation. In addition, a small protein CP12 has also been identified in most of these complexes(141,147). As the formation of this supramolecular complex occurs under oxidizing conditions, the GAPDH/CP12/PRK complex is disassembly by reducing agents such as DTT, which facilitates the purification of isolated PRK and a sub-complex of GAPDH and CP12, also known as native GAPDH (148,149). The interaction between oxidized CP12 and GAPDH provides full protection from oxidative damage (149). In higher plants, chloroplast GAPDH is forms an A<sub>2</sub>B<sub>2</sub> tetramer. The B subunit holds a C-terminal extension responsible for the oligomerization of higher plant chloroplast GAPDH into an A8B8 regulatory form (149,150). In the algae *C. reinhardtii*, the PRI, PRK, PGK, GAPDH, and FBPase co-localize and are located near the thylakoid membrane by the



immunolocalization studies. As the Calvin-Benson cycle of higher plants, these enzymes in the *C. reinhardtii* are also assembled as the supramolecular complexes (151) (Figure 8). In addition, the GAPDH is only present as an A<sub>4</sub> homotetramer in the *C. reinhardtii* and *Scenedesmus obliquus*. Interestingly, the sequence similarity of CP12 with C terminal extension of B subunit of GAPDH, that contains two cysteine residues, believed to be involved in the redox regulation of this enzyme (152). However, the experimental evidence for substrate channeling between Calvin–Benson cycle is lacking, despite the fact the formation of super-complexes is proposed to be related to a regulatory mechanism for CO<sub>2</sub> assimilation.

## 2. Photorespiration

In the central photorespiratory pathway of leaf peroxisomes, the of inner membrane microcompartment of peroxisomes is the site of the possible interaction of the glycolate oxidase (GOX), catalase, Ser-glyoxylate and Glu-glyoxylate aminotransferase, hydroxypyruvate reductase (HPR), and MDH (Figure 8). Moreover, the complex formation and possibility metabolite channeling among the components of glycine decarboxylase system remains unclear. The association-disassociation of glycolate oxidase and catalase depends on dynamic changes in hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) levels (153). In the isolated spinach leaf peroxisomes, the glycerate was produced at the same rate with and without an intact membrane and, in both cases the intermediates glyoxylate and hydroxypyruvate are not detected in the suspension medium, indicating that the possible substrate channeling in the photorespiratory pathway of leaf peroxisomes (154). Assembly and dynamics of these multi-enzyme complexes *in vivo* in peroxisomes may reveal the additional regulatory mechanisms for photorespiration (19). In addition, the channeling of tetrahydrofolate between mitochondrial glycine decarboxylase system T protein and serine hydroxymethyl transferase has been expected due to the sensitivity of tetrahydrofolate to oxidation, however, this was not proved by the *in vitro* enzyme kinetics (155). To conclude, whilst enzyme-enzyme assemblies clearly appear to be prominent in the twin pathways of photosynthesis and photorespiration considerably more study is required to better understand their form and function.

## STRUCTURAL SUPPORT AND NUCLEATION POINTS FOR ENZYME-ENZYME ASSEMBLIES

Unlike oligomeric protein assemblies and stable multi-enzyme complexes, understanding of the regulation of transient enzyme-enzyme assemblies i.e. factors that promote their association/dissociation is currently relatively poor. Indeed until recently it has largely been confined to the demonstrations that lipid microdomains (15,156-158), cytoskeletal elements (43) and carrier proteins (159) could form structural support for enzyme-enzyme assemblies. As described above, the latter two mechanisms have been demonstrated to be involved in enzyme-enzyme assemblies involved in energy and redox metabolism. Similarly, a wide range of phenylpropanoids metabolons have been proposed to be stabilized at the ER membrane (15,16). A further recent hypothesis emanated from the comparative study of cell-free and *E. coli* glycolytic pathways which identified the phosphotransfer system of *E.coli* as a possible nucleation point for the glycolytic metabolon in this species (160,161). Both *in vitro* and *in vivo* experiments proved that the Embden-Meyerhof-Parnas flux between isozymes of phosphofructokinase and fructose-bisphosphate aldolase are channeled and the phosphotransfer system may be an anchor point to initiate enzyme assemblies (161). In addition to the TCA cycle, the interactions between regulatory subunits and catalytic subunits of different proteins act as nucleation points (14), which help in the formation of the specific interaction between the sequential enzymes (10). Thus, the sequential enzymes of metabolic pathway formed the metabolon with help of both by protein-protein interactions and by structural elements of the cell (integral membrane proteins and proteins of the cytoskeleton).

Whilst reports have suggested that phosphorylation disrupts the human carbonic anhydrase metabolon (162), large-scale structure-function studies have yet to be carried out in order to ascertain if this is a common mechanism for the regulation of enzyme-enzyme assemblies. An intriguing recent hypothesis that is receiving growing theoretical and experimental support is that the metabolic intermediates of the pathways themselves are determinants of the complex formation. An early example of this regards the metabolon formation that was observed following the conformational change in cytochrome P450 enzymes on substrate binding which mediates the attachment of cytochrome 450 reductase (15,40). It seems likely that advances in crystallography and

electron microscopy will render such discoveries more common in the near future. Chemists and physicists have recently developed experimental systems for testing enzyme-enzyme assemblies that are based on cell-free systems that either replicate molecular crowding (163) or represent simple systems in which to evaluate enzyme chemotaxis (164). Whilst the former, will undoubtedly provide new insight into the dynamics of cellular metabolism, for reasons described earlier (165) we do not believe that such simulated cells will prove great insight into enzyme-enzyme assemblies. As described above the enzyme chemotaxis studies, on the other hand, provide a novel mechanism by which metabolic intermediates may govern the formation of enzyme-enzyme assemblies has been uncovered by *in vitro* experiments of the effect of metabolite gradients on complex formation. These studies suggest that enzymes show chemotactic movement along their substrate gradient in a manner that could drive their co-localization (63,166-168). Typically this is followed by using fluorophore-tagged enzymes to follow movement in microfluidic devices(19), however, the conclusions made have been critically discussed due to issues in the interpretation of the fluorescence correlation spectra (169). A further issue is whether these devices adequately reflect how the enzymes behave in a cellular system. As such developments of approaches to test this are an utmost priority. These criticisms notwithstanding these findings have inspired chemists and physicists alike to assess these phenomena with many further recent papers being published in this area worthy of mention.

The results of two recent microfluidic experiments are particularly interesting. The first of these used super-resolution fluorescence measurements performed across four orders of magnitude of substrate concentration demonstrated that this dramatically boosted motion providing demonstration that catalytic activity is linked to chemotactic and antichemotactic properties (170). The second study used <sup>13</sup>C labelling in cell free systems to investigate channeling in glycolysis and compare this with *in vivo* studies evidencing considerable advantage in the more channeled system arising in the cell free system (161).

Modelling studies have also focused on the chemotactic movements exhibited by catalytic enzymes and proposing potential mechanisms by which this is achieved with a wide range of possibilities being explored including self-thermophoresis, boosting of kinetic energy,

stochastic swimming and collective heating theories being proposed (167). As yet, there does not appear to be a consensus regarding the most likely mechanism however only modelling a combination of stochastic swimming and collective heating have yet been demonstrated to reproduce changes in diffusion consistent with experimental evidence (171). The picture is complicated yet further by hydrodynamic interactions between the enzyme and the non-uniform substrate with which it interacts (167). However, given the vast interest in these phenomena it would appear likely that considerable advances in this area will be made within the next decade

## CONCLUSIONS

Whilst there is admittedly, on the basis of dissociation constants, no clear distinction between stable multi-enzyme complexes and transient enzyme-enzyme assemblies these complexes would appear to have evolved under different selective pressures to take different roles in metabolic regulation. Whilst there is a huge literature underlying the structure-function relationships of multimeric enzymes as well as multi-enzyme complexes which has led to a detailed understanding of the mechanisms which confer their stability, much less is known concerning this feature for transient enzyme-enzyme assemblies (including metabolons). That said growing support that cytoskeletal or membranous structures provide support or nucleation points for transient enzyme-enzyme assemblies. The nucleation points of the metabolon formation also provide more information to the *in vivo* enzyme cluster engineering in synthetic biology. Moreover, work in the last few years has led to the fascinating hypothesis that such assemblies form due to the presence of concentration gradients of their substrates. Although questions remain regarding the interpretation of the fluorescence signals that these conclusions are based on and the occurrence of such behavior *in vivo*, this is a highly exciting idea that has brought fresh impetus to the study of transient enzyme-enzyme complexes. Provision of *in vivo* evidence of such behavior is clearly a pressing challenge for future research. However, once achieved this, alongside structure-function studies, this will represent a fantastic opportunity to gain deeper understanding of the stability of transient enzyme-enzyme complexes, alongside properties of their association and dissociation.

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## Abbreviations:

TCA cycle: tricarboxylic acid cycle

mtLPD: mitochondrial dihydrolipoyl dehydrogenase

PDH: pyruvate dehydrogenase complex

OGDH: 2-oxoglutarate dehydrogenase

GDC: glycine decarboxylase complex

Y2H: yeast two-hybrid

Spluc: split-luciferase

BRET: bioluminescence resonance energy transfer

FRET: fluorescence/Foerster resonance energy transfer

CLSM: confocal laser scanning microscopy

FLIM-FRET: fluorescence lifetime imaging microscopy based FRET

BioID: proximity dependent biotin identification

APEX: ascorbic acid peroxidase

AP-MS: affinity purification followed with mass spectroscopy

BiFC: bimolecular fluorescence complementation

FAS: fatty acid synthase complex

mETC: mitochondrial electron transport chain complexes

E: enzyme

TAP: tandem affinity purification

Co-IP: Co-immunoprecipitation

TS-DHFR: thymidylate synthase-dihydrofolate reductase

G6P: glucose 6-phosphate

F6P: fructose 6-phosphate

F1: 6BP: fructose 1, 6-bisphosphate

DHAP: Dihydroxyacetone phosphate

GAP: glyceraldehyde 3-phosphate

1,3-BPG: 1, 3-Bisphosphoglycerate

3PG: 3-Phosphoglycerate

HXK: Hexokinase

PGI: Phosphoglucose isomerase

PFK: Phosphofructokinase

ALD: Aldolase

TPI: Triosephosphate isomerase

GAPDH: Glyceraldehyde phosphate dehydrogenase

PGK: Phosphoglycerate Kinase

ENO: Enolase

PK: Pyruvate Kinase.

PDC: pyruvate dehydrogenase complex

ME: malic enzyme

CSY: citrate synthase

ACO: aconitase

IDH: isocitrate

ODC: oxoglutarate dehydrogenase complex

SCoAL: succinyl-CoA ligase

SDH: succinate dehydrogenase

FUM: fumarase

MDH: malate dehydrogenase

2OG: 2-oxoglutarate

CI: Complex I (NADH:ubiquinone oxidoreductase)

CII: Complex II (succinate dehydrogenase)

CIII: Complex III (cytochrome bc1)

CIV: Complex IV (cytochrome c oxidase)

cyt c: cytochrome c.

RPI: ribose-phosphate isomerase

PRK: phosphoribulokinase

RuBPCase: ribulose-bisphosphate carboxylase/oxygenase

PGK: phosphoglycerate kinase

GAPDH: glyceraldehyde-phosphate dehydrogenase

Ru-5-P-K: ribulose-5-phosphate kinase

Sed-1:7-bPase: sedoheptulose-1, 7-bisphosphatase

FNR: ferredoxin-NADP+ reductase

HPR: hydroxypyruvate reductase

H<sub>2</sub>O<sub>2</sub>: hydrogen peroxide

TPI: triphosphate isomerase

FBA: fructose-bisphosphate aldolase

FBPase: Fructose biphosphatase

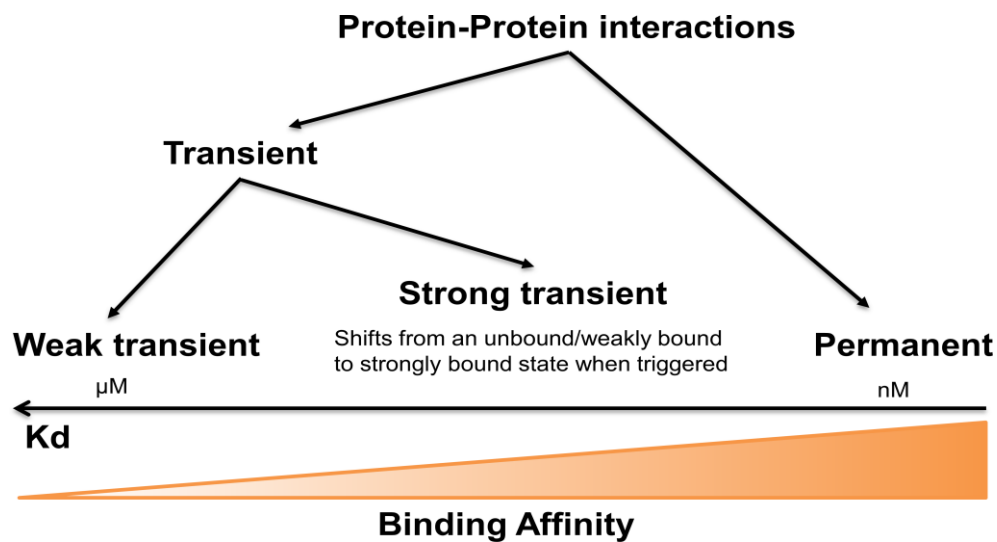
RPE: ribulose-5-phosphate 3-epimerase

SBPase: sedoheptulose-bisphosphatase

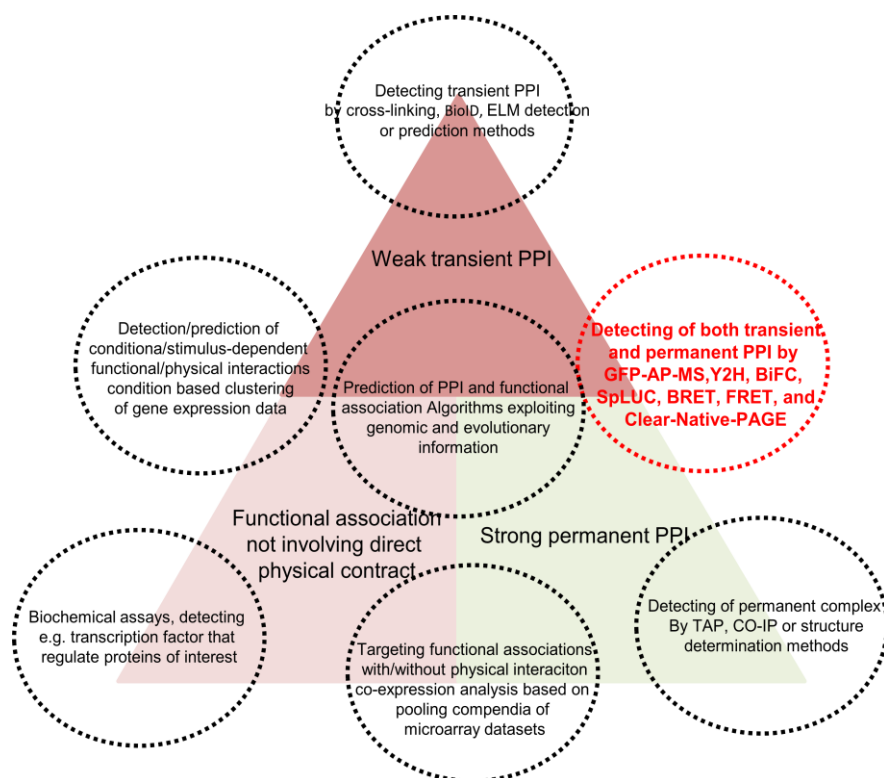
GOX1/2: glycolate oxidase 1/2

GGT1: glutamate:glyoxylate aminotransferase 1

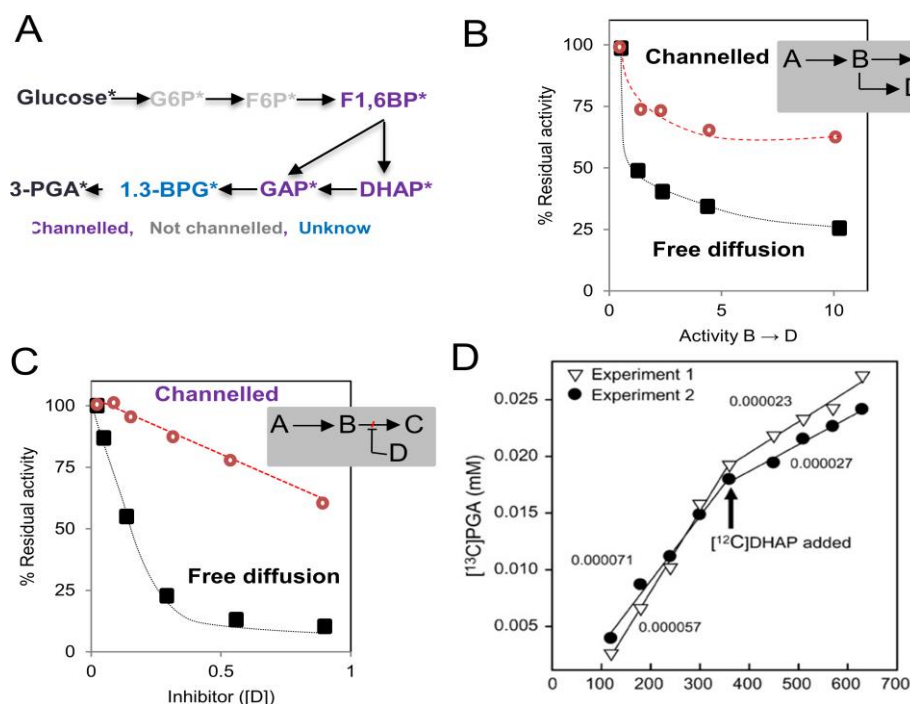
SGAT1: serine:glyoxylate aminotransferase 1



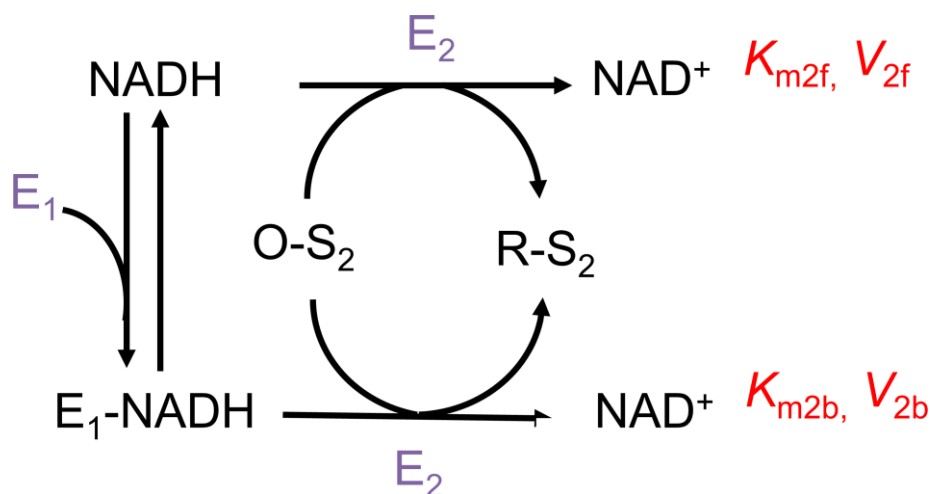
**Figure 1. The binding affinities of different types of protein-protein interactions are inversely related to the dissociation constant ( $k_{\text{off}}/k_{\text{on}}$ )  $K_D$ . The binding affinity is ranged between  $\mu\text{M}$  (weakly transient interaction) and  $\text{nM}$  (permanent interaction) modified from (110).**



**Figure 2. The different type protein-protein interaction detection method based on the binding affinity.** Single approaches can be biased and limited, but when taken together they can provide a more comprehensive view of biological networks **modified from (110)**. Abbreviations: AP-MS, affinity-purification mass spectrometry; Y2H, yeast two hybrid; BiFC, bimolecular fluorescence complementation; Spluc, split-luciferase; BRET, Bioluminescence resonance energy transfer; FRET, fluorescence resonance energy transfer; TAP, tandem affinity purification; Co-IP, Co-immunoprecipitation; BioID, proximity dependent biotin identification.

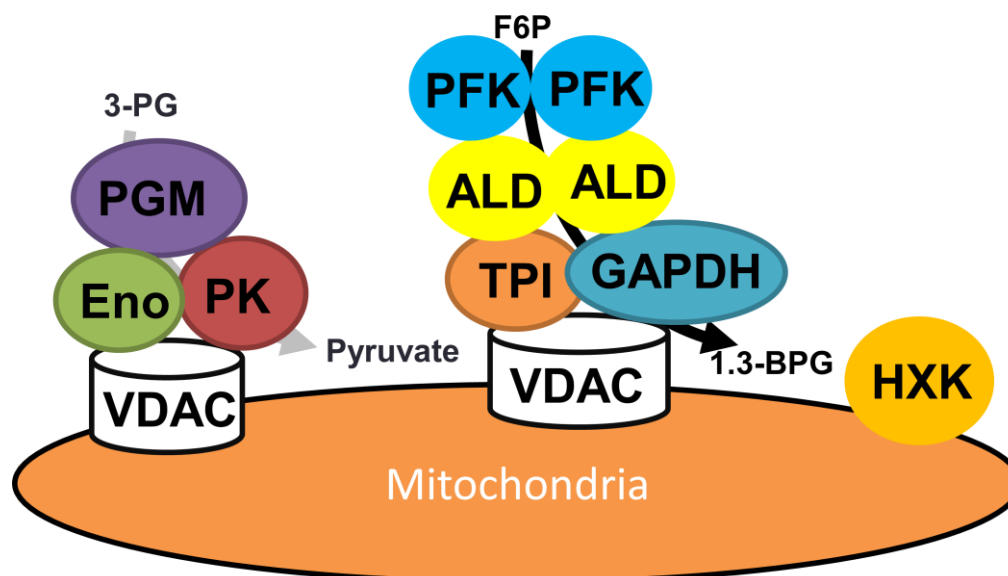


**Figure 3. Methods of evaluating substrate channeling.** A, The isotope dilution experiment was used to test channeling of glycolysis. The isolated potato mitochondria was fed with <sup>13</sup>C labelled glucose and separately added the no-labelled intermediates (G6P, F6P, F1,6BP, DHAP or GAP) to calculate the fractional enrichment in products reaching steady state (42,51). B, A reaction scheme and depiction of transient time (t) analysis is based on data from a channelled bifunctional thymidylate synthase-dihydrofolate reductase (TS-DHFR) and a freely diffusing Monofunctional TS and DHFR (data from (42,147)). C, 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 alanine aminotransferase which competes for the metabolic intermediate (data from (42,147)). D, The result of isotope dilution experiments for DHAP. The time course plots showing the fractional <sup>13</sup>C enrichment in PGA following the addition of DHAP at 0 min. Data come from (42,51). Abbreviations: G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; F1, 6BP, fructose 1,6-bisphosphate; DHAP, Dihydroxyacetone phosphate and GAP, glyceraldehyde 3-phosphate.

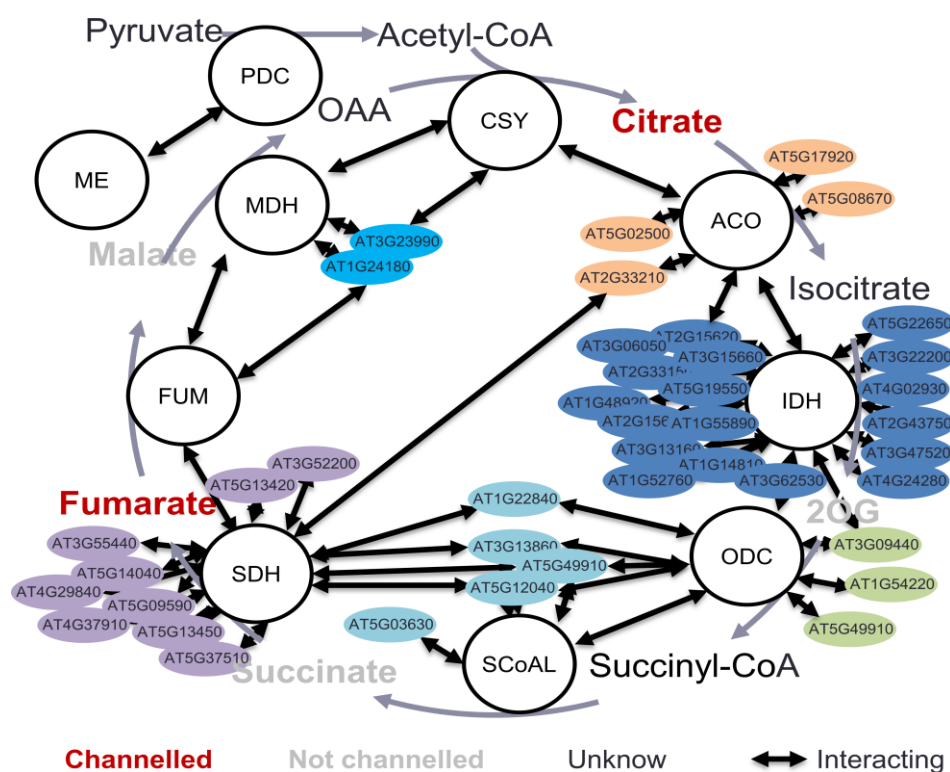


**Figure 4, Enzyme buffering analysis of channeling.** This approach is typically applied for following the channeling of NADH which assesses if the second enzyme of a couple can use bound as well as free NADH and is based on comparison of the reaction velocities following dramatic decreases in the size of the free NADH pools as represented in the scheme. If the enzyme is not able to utilize bound NADH the system is essentially just buffering NADH added to it hence the name modified from (42,147).

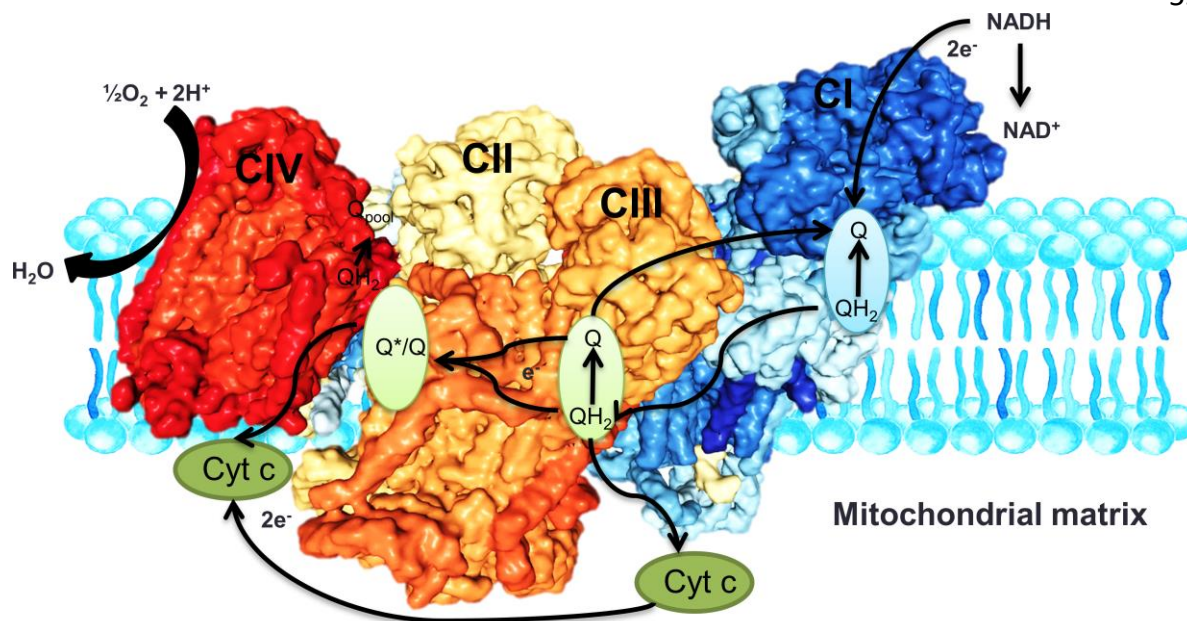




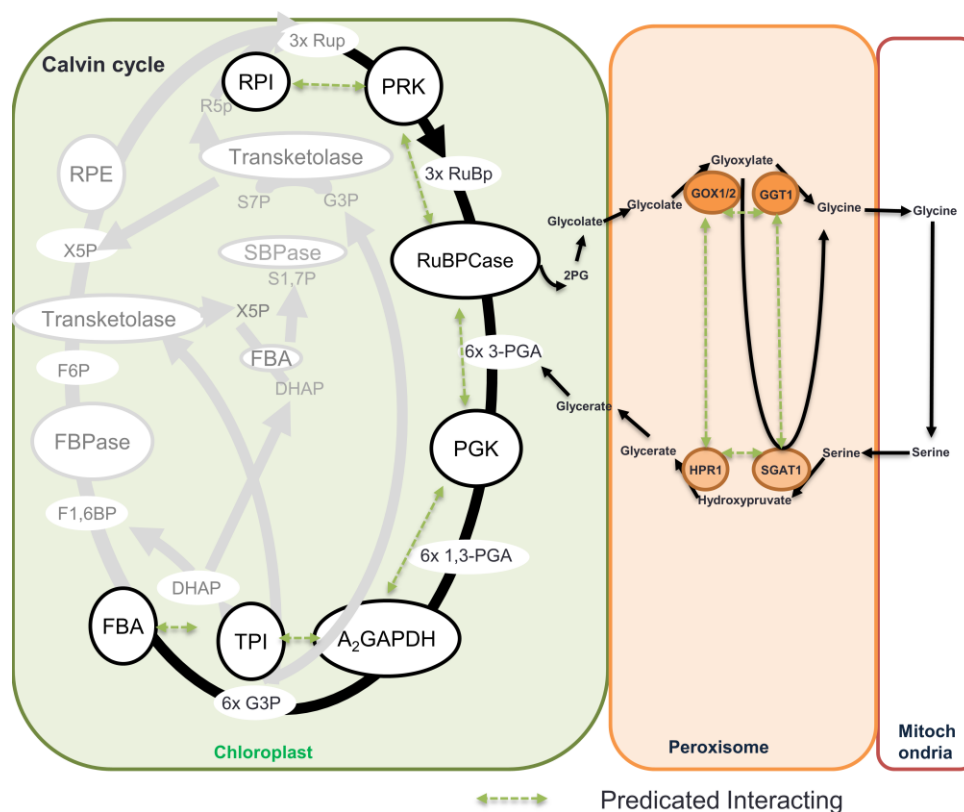
**Figure 5. The protein complex of the glycolysis in outer membrane of mitochondria.** The plant protein complex of catalytic subunits are described next to the arrows (42,51). Abbreviations: 1,3-BPG, 1,3-Bisphosphoglycerate; 3PG, 3-Phosphoglycerate; HXK, Hexokinase; PGI, Phosphoglucose isomerase; PFK, Phosphofructokinase; ALD, Aldolase; TPI, Triphosphosphate isomerase; GAPDH, Glyceraldehyde phosphate dehydrogenase; PGK, Phosphoglycerate Kinase; ENO, Enolase; PK, Pyruvate Kinase.



**Figure 6. The protein-protein interaction of the plant TCA cycle.** The plant protein interactions of catalytic subunits that potentially mediate metabolite channeling are described next to the arrows. The tested 37 interactors associated to the TCA cycle have been described at former research (171,172). Abbreviations: PDC, pyruvate dehydrogenase complex; ME, malic enzyme; CSY, citrate synthase; ACO, aconitase; IDH, isocitrate; ODC, oxoglutarate dehydrogenase complex; SCoAL, succinyl-CoA ligase; SDH, succinate dehydrogenase; FUM, fumarase; MDH, malate dehydrogenase; 2OG, 2-oxoglutarate.



**Figure 7. The supercomplexes structure of the of the mETC (modified from (42,84)).** Two electrons from NADH are transited through complex C I which also reduce Q to QH<sub>2</sub>. Diffusing from the Q-tunnel, QH<sub>2</sub> may enter the proximal Q cavity of C III or may diffuse into the membrane pool. In addition, the QH<sub>2</sub> passes one electron to Cyt c in the intermembrane space and one to Q in the distal cavity, creating the Q• intermediate. Abbreviations: CI, Complex I (NADH:ubiquinone oxidoreductase); CII, Complex II (succinate dehydrogenase); CIII, Complex III (cytochrome bc1); CIV, Complex IV (cytochrome c oxidase); cyt c, cytochrome c.



**Figure 8. The possible calvin cycle and photorespiration complex in the chloroplast and peroxisome.** A potential multi-enzyme complex which comprised several pathway enzymes including RPI, PRK, RuBPCase, PGK, GAPDK, TPI and FBA, has been proposed (50,144). Abbreviations: RPI, ribose-phosphate isomerase ; PRK, phosphoribulokinase; RuBPCase, ribulose-bisphosphate carboxylase/oxygenase; PGK, phosphoglycerate kinase ; A<sub>2</sub>GAPDH, glyceraldehyde-phosphate dehydrogenase ; TPI, triosephosphate isomerase; FBA, fructose-bisphosphate aldolase; FBPase, Fructose bisphosphatase; RPE, ribulose-5-phosphate 3-epimerase; SBPase, sedoheptulose-bisphosphatase; GOX1/2, glycolate oxidase 1/2; GGT1, glutamate:glyoxylate aminotransferase 1; HPR1, hydroxypyruvate reductase 1/2; SGAT1, serine:glyoxylate aminotransferase 1.

**Table 1 Comparison of the different types of enzyme-enzyme assembly**

<b>Metabolic pathway</b>	<b>Multienzyme complexes identification, protein-protein interaction and specific protein – membrane interaction detection</b>	<b>Channeling evidence</b>	<b>Reference</b>
Citric acid cycle	Affinity purification mass spectrometry, Cell biology, protein crystallization, site-directed mutagenesis, kinetic modelling,	isoto dilution experiment	(104,147,164,172)
Glycolysis	Cell biology, affinity purification mass spectrometry	isoto dilution experiment	(10,48,51,113)
Electron transport chain	Enzyme assays in the presence/absence of electron donors and inhibitors and genetically modified super-complexes	-	(74)
Fatty acids oxidation	Protein crystallisation	-	(59,119)
Photosynthesis	Proteomics, protein interaction	-	(50,89,144)
photorespiration	Proteomics	-	(16)
Purine biosynthesis	Cell biology, proteomics, mutant analysis, comparison of enzyme assembly with the rate of flux through the pathway	-	(8,44,109)
Metabolism of amino acids	Protein association studies, site directed mutational analysis	-	(60)
Steroid biosynthesis	enzyme kinetic analysis, co-purification studies, cell fractionation experiments,	Channelling of labelled	(30,65,75)

	co-immunolocalisation	intermediates	
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