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Spatial Organization of Metabolic Enzyme Complexes in Cells

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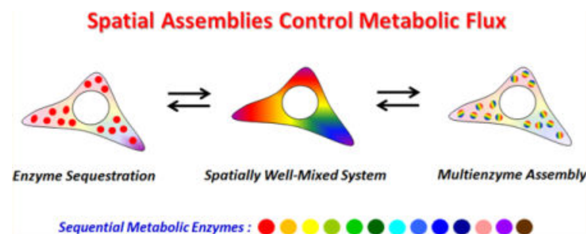
Abstract

The organization of metabolic multienzyme complexes has been hypothesized to benefit metabolic processes and provide a coordinated way for the cell to regulate metabolism. Historically, their existence has been supported by various in *vitro* techniques. However, it is only recently that the existence of metabolic complexes inside living cells has come to light to corroborate this long-standing hypothesis. Indeed, subcellular compartmentalization of metabolic enzymes appears to be widespread and highly regulated. On the other hand, it is still challenging to demonstrate the functional significance of these enzyme complexes in the context of the cellular milieu. In this review, we discuss the current understanding of metabolic enzyme complexes by primarily focusing on central carbon metabolism and closely associated metabolic pathways in a variety of organisms, as well as their regulation and functional contributions to cells.

Graphical abstract



Metabolism is a highly orchestrated process, which provides energy and building blocks to the cell. Typically thought of as a complicated map of hundreds of interconnected chemical reactions, metabolism is key to cellular function, growth, and proliferation. Metabolic pathways are mostly thought to be orchestrated by spatially "well-mixed" enzymes.[1–4] However, this perception has been challenged for many years.[2] Finally, we have begun to understand how metabolic enzymes interact and coordinate with each other in space and time to perform their designed metabolic functions in cells.

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Our understanding of metabolic compartmentalization inside cells has enhanced as advanced cell-based techniques are developed. Systems biology along with omics strategies has found that metabolic enzymes often interact with each other in various compartments of the cell and even demonstrate cell-to-cell variability in protein complex composition.[5–8] In parallel, fluorescence microscopic techniques in association with biochemical and cellular assays have provided compelling evidence of the complexation of metabolic enzymes in cells.[5,9,10] Although subcellular complexation of many enzymes in various metabolic pathways remains to be investigated, it has become clear that metabolism may benefit from the spatial organization of metabolic enzymes in given subcellular locations. Therefore, advancements in cell-based techniques have contributed to our understanding of subcellular localizations of metabolic enzymes and their compartmentalization in cells.

Importantly, the localization of metabolic enzymes into multienzyme complexes can be understood from metabolic and regulatory standpoints. Several metabolic pathways produce and consume chemically unstable or toxic metabolites, so that the proximity of metabolic enzymes to one another would be vital for efficient production of their metabolic products. In addition, spatial and/or temporal concentrations of enzymes and their metabolites are anticipated to generate gradients inside cells.[1,11,12] The association of metabolic enzymes into complexes has been hypothesized to facilitate substrate channeling or influence metabolic flux.[9,13–15] Thus, the localization of metabolic enzymes to or near each other is thought to play a critical role in regulation of metabolic pathways in cells.

In this review, we summarize the current knowledge of metabolic enzyme complexes primarily implicated in the central pathways of carbon metabolism in a variety of organisms (Figure 1, Table 1). Briefly, we discuss the current understanding of the spatial compartmentalization of metabolic enzymes in glycolysis, pyruvate dehydrogenase complex, mitochondrial oxidative phosphorylation, the tricarboxylic acid (TCA) cycle, fatty acid synthesis, and nucleotide biosynthesis. We also describe prospective multienzyme complexes in polyketide biosynthesis and amino acid biosynthesis. However, we do not include extensive knowledge of macromolecular complexes involved in photosynthesis, nucleic acid metabolism, and polypeptide biosynthesis because these complexes have been extensively reviewed.[16–19] Collectively, this review highlights the current status of our understanding of spatial organizations of metabolic enzyme complexes in cells.

GLUCOSE METABOLISM

Glucose metabolism is the central metabolic pathway, which consists of glycolysis and gluconeogenesis. The conversion of glucose to pyruvate in glycolysis is catalyzed in 10 steps, by 10 enzymes, 3 of which are specific to glycolysis. Conversely, gluconeogenesis produces glucose in 11 steps employing four gluconeogenesis-specific enzymes along with seven enzymes from glycolysis (Figure 1A). The compartmentalization of glycolytic and gluconeogenic enzymes in a variety of species has been investigated over many years (Table 2), suggesting that these enzymes would interact and form a multienzyme complex.[20] Here, we review some of the well-characterized protein–protein interactions and their complexes for glucose metabolism in various organisms.

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Glycosome in Protists

The compartmentalization of glycolysis into so-called "glycosomes" has been known for several decades.[21,22] Glycosomes are membrane-bound peroxisomes containing enzymes associated with the first six or seven steps of glycolysis in trypanosmatids.[22,23] To compensate the missing enzymatic activities for glycolysis, trypanosmatids hijack the rest of the glycolytic enzymes from their host organisms to complete glycolysis. The number of glycosomes per cell varies from about 18 to 65, depending on the species.[24,25] Proteomic work has further revealed that, aside from glycolytic enzymes, glycosomes contain various metabolic enzymes in pyruvate metabolism, the TCA cycle, the pentose phosphate pathway, nucleotide metabolism, amino acid metabolism, and steroid metabolism.[26] This suggests that the compartmentalization of glycolysis into glycosomes is a way to coordinate multiple metabolic pathways in protists. Collectively, glycosomes in trypanosmatids are membrane-bound peroxisomes which compartmentalize glycolysis and other pathways to coordinate metabolism.

In addition, glycosomes are essential for trypanosmatids. Abolishment of peroxin proteins, which are required for glycosome formation, resulted in depletion of glycosomes, cytoplasmic localization of the glycolytic enzymes, and cell death, indicating that glycosomes are essential for survival.[27] Without glycosomes, trypanosmatids die in the presence of glucose; thus glycosomes appear to be vital in regulating glucose utilization.[28] Trypanosmatids also regulate the protein composition of the glycosome based on glucose levels,[29] although the glycosome does not appear to govern metabolic flux in trypanosmatids.[30] Collectively, the glycosome appears to be a mechanism of providing metabolic flexibility to trypanosmatids for their survival.[22,30,31]

Glycolytic Complexes in Higher Organisms

Glycolytic enzymes in yeast have been found to be associated with the mitochondria as well as cytoskeletal structures. Specifically, all enzymatic activities of glycolysis have been found in isolated yeast mitochondria.[32] Under hypoxic conditions, enolase in yeast cells was demonstrated to form punctate structures with glucokinase, glucose-6-phosphate isomerase, phosphofructokinase, aldolase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase, and pyruvate kinase.[33] In addition, F-actin was found to provide docking sites for the organization of hexokinase, glucose-6-phosphate isomerase, triose phosphate isomerase, glyceralde-hyde-3-phosphate dehydrogenase, phosphoglycerate mutase, and aldolase in yeast.[34] Particularly, their association with F-actin appears to increase individual enzyme activities while protecting against the inhibitory effects of trehalose in yeast cells.[34] Isotope labeling experiments revealed that the compartmentalization of glycolytic enzymes was associated with increased glucose flux in yeast.[33] Alternatively, in budding yeast cells, phosphofructokinase was recently reported to form cytoplasmic filaments, although the functional relevance of the filament structure remains to be elucidated.[35,36] Collectively, yeast glycolytic enzymes appear to form a variety of subcellular structures, which are responsible for regulating glycolytic flux in cells.

Several proteomic studies with plant cells have also identified that glycolytic enzymes interact with each other as well as with mitochondria.[37–40] In isolated mitochondria from

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Arabidopsis, all 10 glycolytic enzymes were detected by in vitro enzymatic assays, thus indicating that glycolytic enzymes are concurrent with mitochondria.[39,41] The association of glycolytic enzymes to *Arabidopsis* mitochondria suggests the possibility of substrate channeling of metabolic intermediates between cytoplasmic glycolysis and mitochondria.[42] Association or dissociation of various glycolytic enzymes from the mitochondria, including glucose-6-phosphate isomerase, phosphofructokinase, aldolase, triose phosphate isomerase, phosphoglycerate kinase, phosphoglycerate mutase, and pyruvate kinase, was associated with increased or decreased respiration in *Arabidopsis* cells, respectively.[42] Similarly, the association of phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, triose phosphate isomerase, phosphoglycerate kinase, phosphoglycerate mutase, and pyruvate kinase was also promoted when respiration increased in potato tubers.[42] Importantly, glycolytic enzymes associated with mitochondria in *Arabidopsis* appear to be enzymatically active, and their complexation seems to mediate substrate channeling of glycolytic intermediates.[42] Taken together, plant cells promote association of glycolytic enzymes into the multienzyme complex on mitochondria in a cellular respiration-dependent manner, which allows metabolic intermediates to channel through glycolysis into mitochondria.

Glycolytic enzymes are also compartmentalized in *Drosophila* flight muscle cells and *Caenorhabditis elegans* neurons due to their high energy demand. In *Drosophila* flight muscle, glyceraldehyde-3-phosphate dehydrogenase, aldolase, triose phosphate isomerase, phosphoglycerate kinase, and phosphoglycerate mutase have been individually shown to be localized to the sarcomere of myofibrils.[43] However, colocalization or spatial organization of these glycolytic enzymes has not been demonstrated yet in *Drosophila* flight muscle cells. Interestingly, when the first enzyme in the pentose phosphate pathway, glycerol-3-phosphate dehydrogenase, was knocked out, these glycolytic enzymes did not localize to the sarcomere in *Drosophila*.[44] In addition, in *C. elegans*, phosphofructokinase 1.1, glyceraldehyde-3-phosphate dehydrogenase, and aldolase colocalized in clusters at presynaptic sites in neurons under hypoxia or neuronal stimulation.[45] The localization of phosphofructokinase 1.1 into clusters at presynaptic sites was then hypothesized to be due to energy demands for ATP, and the localization, not necessarily enzymatic activities, of glycolytic enzymes to the presynaptic sites seems to be essential for synaptic function.[45] Taken all together, it appears that glycolytic enzyme complexes exist to meet cellular energy needs in high-energy demanding cells, such as *Drosophila* flight muscle cells and *C. elegans* neurons.

The formation of a glycolytic complex has also been investigated extensively in mammalian erythrocytes. Aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphofructokinase, and pyruvate kinase have been found colocalized to the inner surface of the cell membrane of human erythrocytes, in an association with the membrane-bound band 3 protein.[46,47] Mouse glycolytic enzymes were found also colocalized to the membrane of mouse erythrocytes with band 3, despite lacking the conserved sequences between mouse and human band 3 proteins.[48] Furthermore, the glycolytic enzyme complex in human erythrocytes was shown to interact with β-spectrin, ankyrin, actin, and protein 4.2, indicating that glycolytic enzymes are associated with other nonmetabolic proteins in erythrocytes.[49] The association and dissociation of glycolytic enzymes into complexes in mammalian erythrocytes also depended on the oxygenation state of red blood cells as well as the phosphorylation status of band 3.[48,50,51] The formation of a glycolytic complex on the

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erythrocyte membrane is now hypothesized to compartmentalize ATP production in red blood cells.[49] Collectively, work thus far in mammalian red blood cells demonstrates the presence of a multienzyme glycolytic complex, but its functional and/or structural significance remains to be further elucidated.

Aside from glycolytic assemblies in erythrocytes, there is developing evidence for glycolytic enzyme complexes in other human cell types. In HeLa cells, subcellular compartmentalization of glyceraldehyde-3-phosphate dehydrogenase appears to be regulated by small ubiquitin related modifier-1 under hypoxic conditions.[52] More recently, in various cancer cells, we have demonstrated the formation of a multienzyme metabolic complex in the cytoplasm, termed the "glucosome," which contains not only glycolytic enzymes but also gluconeogenic enzymes: phosphofructokinase 1, pyruvate kinase, fructose-1,6-bisphosphatase, and phosphoenolpyruvate carboxykinase.[53] Interestingly, the functional contributions of glucosome clusters to cellular metabolism appear to be differentiated in a cluster-size dependent fashion. Although in-depth studies may be required, the compartmentalization of glycolytic and gluconeogenic enzymes is hypothesized as a mechanism of glucose flux regulation in human cells.

Collectively, it is important to note here that the described data support the existence of multienzyme metabolic complexes for glycolysis in nature. However, most of the described studies have relied on chemically fixed cells and/or in vitro enzymatic assays from chromatographically fractioned pools of cell lysates or semipurified organelle fractions. Also, the protein components of the identified complexes are mostly from glycolysis, thus excluding gluconeogenic enzymes for the pathway. Evidence has indicated that glycolytic complexes may contain more than the pathway enzymes, which may explain their potential regulatory and/or functional actions in cells. It is clear that there are many challenges still ahead to explore new dimensions of glycolytic enzymes and their complexes inside living cells, which will accelerate our endeavors to determine the functional and/or structural significance of such glycolytic complexes in cells.

MITOCHONDRIAL METABOLISM

Pyruvate Dehydrogenase Complex

Under aerobic conditions, the pyruvate dehydrogenase complex (PDC) catalyzes the decarboxylation of pyruvate to produce acetyl-CoA in the mitochondria (Figure 1A). Three essential enzymes, pyruvate dehydrogenase, dihydrolipoamide acetyltransferase and dihydrolipoamide dehydrogenase, associate with each other to form a multienzyme complex 4.5–9 MDa in size.[54] Electron microscopic data have revealed that the PDC is composed of a core of eight dihydrolipoamide acetyltransferase trimers arranged in either a cube (in *Escherichia coli)* or pentagonal dodecahedron (in eukaryotes and some Grampositive bacteria) and is surrounded by 20–30 pyruvate dehydrogenase heterotetramers and 6–12 dihydrolipoamide dehydrogenase homodimers.[55,56] Enzymatically, the lipoyl domain on dihydrolipoamide acetyltransferase plays an essential role by tethering metabolic substrates during the catalytic cycle of acetyl-CoA synthesis. Therefore, extensive in vitro studies have given great understanding to the enzymatic mechanisms and structures of the PDC.

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In addition, a specific set of pyruvate dehydrogenase kinases and phosphatases has been identified to regulate the enzymatic activity of PDC in response to metabolic demand.[54,57,58] The kinase-mediated phosphorylation of the pyruvate dehydrogenase domain deactivates PDC, whereas the phosphatases reciprocally activates PDC by dephosphorylation in the mitochondria. The activities of the kinases and the phosphatases are found to be thermodynamically regulated by the concentrations of PDC's substrate, products, cofactors, and hormones like insulin.[59,60] Using pharmacological inhibitors of the kinases and phospho-specific antibodies for PDC, the phosphorylation-dependent regulation of mitochondrial PDC activity was visualized in fixed mammalian cells under immunofluorescence microscopy.[61] Hence, the PDC activity is under the regulation of a specific set of kinases and phosphatases in cells.

Aside from the mitochondrial matrix, PDC has also been identified in other cellular spaces. First, PDC was found in the mitochondrial outer membrane and intermembrane space, rather than the mitochondrial matrix, in some cancer cells.[62] Second, PDC also translocates from the mitochondria to the nucleus in human cells, although the mechanism of transport remains elusive.[63,64] Interestingly, nuclear-localized PDC seems to catalyze the formation of acetyl-CoA and influences histone acetylation.[63] Therefore, PDC appears to respond to the localized need for metabolites within a cell.

Tricarboxylic Acid Cycle

The tricarboxylic acid (TCA) cycle, also referred to as the citric acid cycle or the Krebs cycle, utilizes two carbon atoms from acetyl-CoA (typically derived from carbohydrates, fatty acids, and amino acids) to generate three molecules of NADH, one molecule of $FADH_2$, and two molecules of CO_2 (Figure 1A). This process is essential to aerobic respiration because NADH and $FADH_2$ are required for ATP production in mitochondrial oxidative phosphorylation. As the TCA cycle requires the coordination of eight enzymes, and occurs in the highly crowded environment of the mitochondrial matrix, it has long been hypothesized that the enzymes of the TCA cycle form a multienzyme complex.[20]

To date, extensive evidence has suggested the existence of a TCA cycle multienzyme complex. Initial in vitro cross-linking studies had found citrate synthase interacted with mitochondrial malate dehydrogenase.[65] The enhancement of enzymatic activities of the TCA cycle enzymes were also identified in gently disrupted rat liver mitochondria, compared to that observed in completely disrupted mitochondria, suggesting the compartmentalization of TCA cycle enzymes in the mitochondria.[66] More recently, intracellular cross-linking techniques and bacterial two-hybrid studies revealed that three enzymes of the TCA cycle in *Bacillus subtilis* (i.e., citrate synthase, isocitrate dehydrogenase, and malate dehydrogenase) form a core multienzyme complex.[67] Additionally, the core complex interacts further with fumarase, aconitase, and succinyl-CoA synthetase through malate dehydrogenase.[67] Isocitrate dehydrogenase of the core complex also interacts with a 2-oxoglutarate dehydrogenase complex.[67] In *Pseudomonas aeruginosa*, a TCA cycle multienzyme complex containing citrate synthase, isocitrate dehydrogenase, malate dehydrogenase, fumarase, aconitase, and succinyl thiokinase was purified by size-exclusion chromatography, and individual enzyme composition was confirmed by in vitro

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enzyme activity assays.[68] In mammalian cells, fluorescence recovery after photobleaching (FRAP) experiments using green fluorescent protein-tagged enzymes revealed citrate synthase (51 kDa), isocitrate dehydrogenase (46 kDa), malate dehydrogenase (36 kDa), and succinyl-CoA synthetase (50 kDa) to have similar apparent diffusion coefficients.[69] Considering these enzymes have different multimeric states and sizes, these data support the potential formation of a four enzyme complex in live cells.[69] Furthermore, tandem mass spectrometric analysis with cross-linked beef heart mitochondria revealed that all enzymes involved in the TCA cycle were indeed found to be associated with each other, with the strongest interaction between malate dehydrogenase and citrate synthase.[70] Collectively, all the evidence strongly supports the association of TCA cycle enzymes into a multienzyme complex in mitochondria.

In addition, the protein-protein interactions or complex formation of the TCA cycle enzymes have been hypothesized to be beneficial for regulating flux through metabolic substrate channeling.[20] In vitro microfluidic studies investigating the free diffusion of malate dehydrogenase and citrate synthase demonstrated that the apparent diffusion coefficients of malate dehydrogenase and citrate synthase were influenced by substrate availability.[71] Importantly, the rate of citrate production did not change when the TCA cycle enzymes were challenged in vitro with other enzymes using the same substrate, indicating limited free diffusion of metabolic intermediates, i.e., substrate channeling.[72] The protein-protein interaction between malate dehydrogenase and isocitrate dehydrogenase was also strengthened by the addition of cofactors and substrates of isocitrate dehydrogenase.[73] Taken all together, these data support the hypothesis that a multienzyme complex of the TCA cycle enzymes promotes substrate channeling during the TCA cycle.

Mitochondrial Oxidative Phosphorylation

Mitochondria are often considered as the “powerhouse of the cell” referring to the process of oxidative phosphorylation, which occurs within the inner mitochondrial membrane. In oxidative phosphorylation, the production of ATP is coupled to the generation of a proton gradient via the electron transport chain (ETC) organization. The ETC organization is composed of four macromolecular complexes, termed complexes I, II, III, and IV, coupled with coenzyme Q and cytochrome *c*. The organization of the ETC has been rigorously investigated, as discussed herein.

Investigations into the compartmentalization of the ETC have established the formation of a “supercomplex”. Initially, the four complexes of the ETC and ATP synthase were thought to be randomly distributed throughout the mitochondrial membrane, and oxidative phosphorylation occurred in a random collision model.[74] However, in vitro evidence supported the formation of a supercomplex of the ETC, which is composed of complexes I, III, and IV in mammals, or complexes III and IV in *Saccharomyces cerevisiae*.[75] The mammalian supercomplex model was further strengthened by cryoelectron microscopy visualizing its architecture from sheep heart mitochondria.[76] The data were also confirmed by crosslinking studies in mice mitochondria.[77] Additionally, the association between complex III and complex IV was found to be mediated by supercomplex assembly factor I.[78] Noticeably, complex II is excluded from the respiratory supercomplex; however the reason

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for this has not been investigated yet.[79] Therefore, the compartmentalization of the complexes into a supercomplex is conserved across species for oxidative phosphorylation.

Along with genetic and cryoelectron microscopic techniques, proteomic mapping techniques have recently advanced our understanding of the ETC-ATP synthase organization in mitochondria of living cells. In this technique, live human cells were transfected with mitochondria-targeted ascorbate peroxidase and then treated with biotin-conjugated phenol in the presence of hydrogen peroxide, followed by chemical fixation and pull-down of biotin-labeled protein components for tandem mass spectrometry.[6] The portions of the ETC facing either the intermembrane space or mitochondrial matrix were successfully mapped to visualize the orientation of the ETC organization in human mitochondria.[6,80] This technique has also been used on a larger scale in *Drosophila* to profile mitochondria-associated proteins, including the components of the ETC.[6,80,81] Collectively, spatial and lateral orientations of protein subunits of the ETC organization corroborate our current understanding of the ETC organization in mammalian mitochondria.

While a clear picture of the ETC supercomplex has come into view, the biological purpose of the supercomplex is less understood. Studies conflict as to whether the supercomplex is functionally capable of oxidative phosphorylation.[82–84] On the other hand, various alternative functions for the supercomplex have been proposed, such as substrate channeling or limiting the generation of reactive oxygen species.[74] Thus, functional characterization of the ETC supercomplex inside cells largely remains elusive.

NUCLEOTIDE BIOSYNTHESIS

Purine Biosynthesis

Purine nucleotides are essential molecules, used in the cell for a variety of purposes such as DNA and RNA metabolism, cell signaling, and cellular energetics. In rapidly growing cells, the biosynthesis of purine nucleotides is fully promoted via a salvage pathway as well as a de novo pathway. The latter 10-step process is catalyzed by six enzymes including three multifunctional enzymes in human cells, converting phosphoribosyl pyrophosphate into inosine monophosphate (Figure 1A). Interestingly, one enzyme, trifunctional glycinamide ribonucleotide transformylase, catalyzes three nonsequential steps of de novo purine biosynthesis, suggesting that the enzymes in de novo purine biosynthesis may interact with each other and thus form a multienzyme complex to control purine flux.[85] Indeed, this hypothesis was initially supported by copurification experiments revealing the activities of multiple purine biosynthetic enzymes in the same fraction of tissue extracts.[86,87] However, it has been difficult to experimentally demonstrate direct protein–protein interactions or complex formation in vitro among the pathway enzymes. Nevertheless, earlier experimental data have suggested the potential existence of multienzyme complexes for this pathway in cells.

Recently, a metabolic complex of purine biosynthetic enzymes, namely, the “purinosome,” was identified in living human cells under purine deprivation.[88] The reversible nature of purinosome assemblies was demonstrated in response to purine levels, indicating their functional contribution to the cells.[88] Later, purinosome formation was positively correlated

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with increased levels of purine metabolites, compared to cells lacking purinosomes, thus corroborating the metabolic activity of purinosomes in live cells.[89,90] Biochemical and biophysical studies have also proposed that three enzymes involved in the first half of the pathway (steps 1–5) form a core structure of the purinosome, while the other three enzymes, catalyzing steps 6 through 10, are dynamically associated with the core complex via protein–protein interactions.[91,92] Furthermore, knockout of any purine biosynthetic enzyme resulted in either the reduction or abolishment of purinosome association in human cells.[93] More recently, the functional activity of mechanistic target of rapamycin (mTOR) was linked to the spatial association of purinosomes with the mitochondria as well as purine biosynthesis.[94,95] Collectively, the formation of purinosomes, which indicates the upregulation of de novo purine biosynthesis, has significantly advanced our understanding of the regulatory mechanisms of de novo purine biosynthesis in human cells.[96–98]

In addition, we have recently identified a sequestrationmediated downregulation mechanism of de novo purine biosynthesis. The basal level activity of de novo purine biosynthesis has been detected in the absence of purinosome assemblies or under conditions in which purinosome formation was not favorable.[89,90,99] These data suggest that purinosomenegative cells maintain a certain level of metabolic activity of de novo purine biosynthesis, and questions if de novo purine biosynthesis is downregulated in human cells. Excitingly, we have identified that AMP-dependent protein kinase (AMPK) promotes the spatial sequestration of one of the purinosome core enzymes into its own self-assemblies for downregulation of de novo purine biosynthesis in HeLa cells.[100] Therefore, it has become clear that spatial assemblies of purine biosynthetic enzymes can regulate the metabolic activity of purine metabolism in living human cells.

Furthermore, other enzymes in purine metabolism appear to form cytoplasmic structures in cells. Inosine monophosphate dehydrogenase 2 (IMPDH2) and adenylosuccinate synthase were demonstrated to be part of the purinosome clusters in HeLa cells, indicating the participation of other purine enzymes in the purinosome assembly.[90] Alternatively, IMPDH2 was shown to form cytoplasmic rod and ring structures in the cytoplasm of various mammalian cells.[101–104] Such rod and ring structures of IMPDH2 were promoted in mammalian cells by glutamine, serine, or glycine starvation.[105,106] Conversely, however, the cytoplasmic structures of IMPDH were found to dissociate in mouse pancreatic islets, but not in other tissues, under fasting conditions in mice.[103] Collectively, the enzymes which catalyze de novo adenine and guanine biosynthesis appear to be associated with the purinosome, while some are capable of forming their own independent cytoplasmic structures.

Pyrimidine Biosynthesis

De novo pyrimidine biosynthesis is a nine-step pathway catalyzing the conversion of L-glutamine to CTP, utilizing four enzymes, two of which are multifunctional (Figure 1B). Unlike de novo purine biosynthesis, the formation of a sequential multienzyme complex catalyzing pyrimidine biosynthesis has not been systematically investigated, likely because the enzymes are not all cytoplasmic.[107] However, there is evidence for the oligomerization of single enzymes involved in pyrimidine biosynthesis.

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The multifunctional enzyme catalyzing the first three steps of pyrimidine biosynthesis, with carbamoyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase (CAD) activities, has been found to form clusters within mammalian cells.[96,108–110] The formation of CAD clusters is phosphorylation-dependent.[108] Specifically, active mTORC1 has been found to promote the clustering of CAD through ribosomal protein S6 kinase beta-1, resulting in the upregulation of pyrimidine biosynthesis.[109–111] Therefore, the compartmentalization of CAD, controlled by post translational modifications, appears to control pyrimidine biosynthesis.

There have also been extensive investigations into the spatial assembly of cytidine triphosphate synthase (CTPS), which catalyzes the final step of pyrimidine biosynthesis. Briefly, CTPS has been shown to form filament structures in bacteria, fly, yeast, and human cells.[102,112–118] *In vitro* investigations of the filament structure of *E. coli* CTPS have found that CTPS is assembled into tetramers stacked upon each other to promote bacteria curvature as cytoskeletal elements.[113,119] In addition, the expression of the transcription factor Myc is positively associated with induction of CTPS filaments, while nonreceptor tyrosine kinase Ack appears to control the formation of CTPS filaments to regulate CTPS activity.[120,121] Direct ubiquitination of CTPS seems to be negatively associated with the formation of CTPS filaments in *Drosophila* and human cell lines.[122,123] Nevertheless, it appears that the CTPS filaments are composed of metabolically inactive CTPS.[119] This notion was further corroborated by evidence that the formation of CTPS filaments was induced by inhibition of CTPS activity in *Drosophila* and various vertebrate tissues including human cancer cells.[102,115,124] Therefore, it will be interesting to investigate how the other mitochondrial enzyme involved in *de novo* pyrimidine biosynthesis spatially and/or temporally perform sequential reactions with the cytoplasmic structures of CAD or CTPS in the cell.

LIPID METABOLISM

Fatty Acid Synthesis

Fatty acid synthesis is the process in which the cell utilizes carbohydrates to make fatty acids for lipid membranes, protein modifications, hormone synthesis, and energy storage. In fatty acid synthesis, an activated acetate or an acyl chain is extended by two carbons in four steps, and repeated until forming a chain containing 16 or 18 carbon units (Figure 1A). All the steps in fatty acid synthesis are catalyzed by a multifunctional fatty acid synthase (FAS), which can range in size from 540 kDa to 2.6 MDa.[125] Interestingly, type I FAS, expressed in mammals and fungi, contains all enzymatic activities of fatty acid synthesis on one or two polypeptide chains. On the other hand, prokaryotes, chloroplasts, and mitochondria express type II FAS, where each step of fatty acid synthesis is performed by an individual enzyme, requiring the coordination of those enzymes. Since this system has been extensively reviewed recently,[125–127] we focus on recent investigations into the structure and the cellular compartmentalization of FAS, which have shed light on the process of fatty acid synthesis.

Essential to fatty acid synthesis is the movement of the growing acyl chain from one active site to the next. Structures of FAS from *Thermomyces lanuginosus* and *S. cerevisiae* have revealed that FAS contains a “reaction chamber” in which the acyl carrier protein shuttles the substrate from one active site to the other.[128–130] Single particle cryoelectron microscopy

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has further revealed a variety of conformations of rat FAS, which support dynamic conformational changes of FAS during fatty acid synthesis.[131] Thus, the macromolecular structure of FAS provides a mechanistic insight of how recurring sequential reactions are orchestrated from one active site to the other between multiple enzymatic activities.

In addition, the subcellular location of FAS has been investigated along with other lipid biosynthetic enzymes in cells. In yeast, fatty acid synthesis is accomplished by two subunits, Fas1 and Fas2, which colocalize into cytoplasmic clusters in the quiescence stage of growth as visualized by fluorescence microscopy.[132] The FAS sequestered into clusters retains its activity, indicating the clusters are not aggregates or misfolded proteins.[132] Glucose starvation in yeast also independently promoted the clustering of other biosynthetic enzymes utilizing fatty acids, including acetyl-CoA carboxylase in the cytoplasm, phosphatidylinositol synthase in the endoplasmic reticulum, and phosphatidylserine decarboxylase in the mitochondria.[132] Furthermore, in human cells, FAS was identified to interact with ATP-citrate lyase and fatty acid transporters on the peroxisome membrane by bioluminescence resonance energy transfer.[133] Therefore, these subcellular localization studies indicate the potential dynamics of FAS to localize with various cellular compartments.

NATURAL PRODUCT BIOSYNTHESIS

Polyketide and Non-Ribosomal Peptide Synthesis

Several natural products, which are commonly used as antibacterials, antifungals, or toxins, are biosynthetically synthesized by polyketide synthases or nonribosomal peptide synthetases, utilizing carboxylic acids or amino acids, respectively.[134–138] Like fatty acid synthesis, the biosynthesis of such natural products occurs in a linear fashion by one macromolecular complex containing multiple active sites, or through sequential reactions of many smaller enzymes. Given the mechanistic similarity between fatty acid synthesis and natural product biosynthesis, we briefly summarize here the subcellular localization of metabolic enzymes involved in natural product biosynthesis.

In bacteria, natural product metabolism often occurs in membrane-bound vesicles. For example, in *Aspergillus fumigatus* and *Aspergillus nidulans*, enzymes involved in the early steps of melanin biosynthesis are localized to endosomes, whereas enzymes involved in the later steps of the synthesis are localized to the cell wall by the palmitoylation of the enzymes.[139,140] Furthermore, these enzymes interact with each other in their respective subcellular locations, possibly allowing substrate channeling for melanin biosynthesis. A similar strategy localizing biosynthetic enzymes in vesicles is also used to synthesize the toxin aflatoxin in *Aspergillus parasiticus*.[141,142] The number of the aflatoxin-synthesizing vesicles is increased in response to the promotion of aflatoxin biosynthesis, followed by the transportation of aflatoxin out of the cell by exocytosis.[143] Thus, some organisms have a mechanism to organize biosynthetic enzymes into lipid vesicles for biosynthesis and secretion of complex biomolecules.

In contrast to the vesicle-mediated biosynthesis of natural products, several species of bacteria appear to produce natural products in large nonmembrane bound enzyme

complexes. For example, *Pseudomonas aeruginosa* promotes localization of biosynthetic enzymes involved in siderophore biosynthesis to the cell membrane.[144,145] The enzyme complex, so-called "siderosome," is membrane-associated at the bacterial poles at the early exponential phase of growth through weak protein–protein interactions.[144] Since siderophores are essential biomolecules for pathologic bacteria, and serve as iron scavengers for cellular function, it appears that cells do not need to export these biomolecules through the vesicle-mediated exocytosis. In addition, the natural product bacillaene in *Bacillus subtilis* is produced by a large (~2.5 MDa) hybrid non-ribosomal peptide synthetase and polyketide synthase enzyme complex.[146,147] The enzyme complex does not appear to be membrane-bound, but fluorescence microscopy and transmission electron microscopy revealed the complex to associate near the cell membrane.[146] Taken together, these examples indicate that enzymes in natural product biosynthesis form spatial organizations for efficient regulation of their metabolic products in cells.

AMINO ACID METABOLISM

Amino acids serve as the building blocks of peptides and proteins, as well as metabolic intermediates. The 20 canonical amino acids can be biosynthetically made by bacteria and plants, nine of which are essential amino acids for mammals. While the knowledge of amino acid metabolism has spanned decades, we are just now beginning to understand how amino acid metabolism is compartmentalized in the cell.[148–150] Herein, we review evidence for prospective amino acid metabolic multienzyme complexes, which have yet to be clearly defined.

The biosynthesis of aromatic amino acids in plants, prokaryotes, ascomycete fungi, and apicomplexans is accomplished by the shikimate pathway.[151] The shikimate pathway is composed of seven reactions catalyzed by seven enzymes, converting phosphoenolpyruvate and erythrose-4-phosphate to chorismate.[148,152] Chorismate is a precursor to the aromatic amino acids, including phenylalanine, tryptophan, and tyrosine. This pathway has been extensively studied in *Mycobacterium tuberculosis*, wherein the direct interaction between 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase and chorismate mutase, which catalyze the first and seventh steps of the pathway, significantly increased their metabolic activities compared to the enzymes alone.[153,154] Crystal structures of DAHP synthase interacting with chorismate mutase revealed that the binding of tryptophan and phenylalanine to DAHP synthase downregulates the activities of both enzymes in the complex.[155] Although these enzymes do not catalyze sequential steps, their interaction appears to be vital to the production of aromatic amino acids. It seems that the protein-protein interaction may provide the basis for an interaction of all the enzymes in the shikimate pathway and ultimately other enzymes involved in aromatic amino acid biosynthesis.

The catabolism of the essential branched chain amino acids, leucine, isoleucine, and valine, is accomplished by the branched-chain α-keto acid dehydrogenase complex. The protein complex is composed of a core of dihydrolipoyl transacylase subunits, associated with branched-chain α-keto acid decarboxylase/dehydrogenase, dihydrolipoamide dehydrogenase, branched-chain α-keto acid dehydrogenase complex kinase and

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phosphatase.[156] Affinity chromatographic studies investigating other proteins associated with the complex in mammalian systems revealed weak protein-protein interactions between the branched-chain α-keto acid decarboxylase/dehydrogenase component of the complex and mitochondrial B_6-dependent branched chain aminotransferase.[156] Furthermore, this interaction increases the decarboxylation of branched-chain α-keto acids, possibly through substrate channeling. Meanwhile, glutamate dehydrogenase and pyruvate carboxylase are also found to be the components of the so called "branched-chain amino acid metabolon".[157] The binding of glutamate dehydrogenase to the metabolon resulted in more efficient channeling of products to oxidative pathways, proposing a functional role of the metabolon in branched chain amino acid catabolism.[157] Therefore, the compartmentalization of amino acid catabolism has been hypothesized to promote substrate channeling.

CONCLUDING REMARKS

Metabolism is accomplished through the spatial compartmentalization of metabolic enzymes into vesicles, membranes, cellular organelles, or nonmembrane bound cellular granules in the cytoplasm. As it is reviewed in this article, there is extensive evidence for the formation of metabolic complexes in nature. Along with the rich history studying metabolic enzymes and their complexes in vitro, our understanding of the intracellular compartmentalization of metabolism has significantly advanced in recent years due to the advancement of intracellular biochemical and biophysical techniques. Of particular, recent endeavors on de novo purine biosynthesis in living human cells have shed light on the paradigm that "spatial assemblies of sequential metabolic enzymes can regulate metabolic activities of the pathway in living cells".[88,100]

However, our understanding of each multienzyme complex and its functional contributions to cell metabolism is mostly at its infancy. The existence of multienzyme complexes has long been thought to facilitate in substrate channeling and thus influence metabolism.[20,86,87,158,159] By localizing active sites close to one another, sequential metabolic enzymes may benefit to not only increase metabolic efficiency, but also limit the diffusion of toxic or unstable intermediates.[13–15,40,160–162] However, it has been challenging to structurally and kinetically demonstrate such substrate channels among more than three sequential enzymes. Alternatively, cluster-mediated channeling has been recently proposed to explain the metabolic benefit of the spatial assembly of sequential metabolic enzymes in cells.[14,163] In this case, rather than physical coordination between active sites, only colocalization of sequential enzymes seems to be enough to promote metabolic efficiency.[14,163] On the other hand, it is also important to note here that metabolic enzyme complexes have been formed to play as intracellular depot systems, demonstrating that the formation of metabolic complex is not the direct indication of metabolic flux enhancement.[164] Various spatial assemblies are also formed by single enzymes or only a subset of enzymes of given pathways, further indicating potential functional diversities of spatial metabolic assemblies in cells beyond flux enhancement.[35,100,119] It is clear that there is still much to be learned about biological significance of the compartmentalization of metabolic enzymes in cells.

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Collectively, a combination of in vitro and intracellular investigations of metabolic enzymes and their complexes will accelerate our endeavors to understand the functional and structural significance of metabolic organizations and their regulatory mechanisms in the context of the cellular milieu. Ultimately, in vitro and cellular biochemistry will provide the fundamental principles of how metabolism is orchestrated in space and time at molecular levels. This new level of understanding will divulge the importance of heretofore unrecognized metabolic compartments as novel targets for therapeutic intervention, thereby contributing to public health and welfare.

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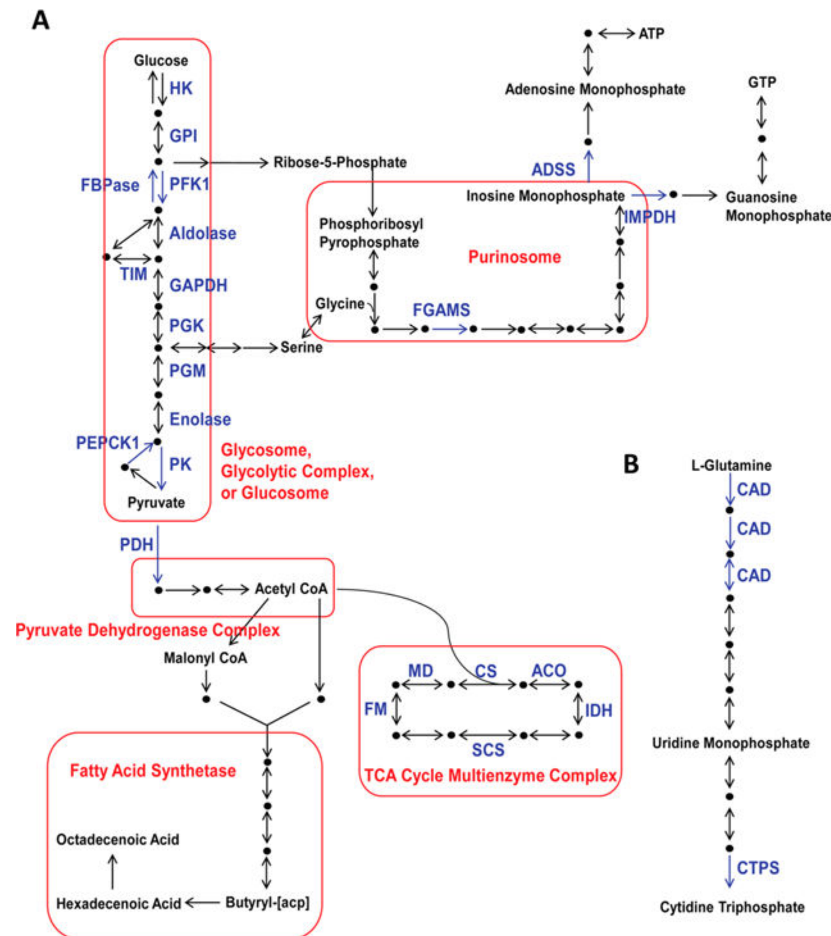

Figure 1.
An overview of central carbon metabolism. Enzymes extensively discussed in this review are named in blue, while discussed multienzyme complexes are boxed in red. Metabolites are either shown by their names or black dots. (A) Glucose is consumed through the central pathway of glycolysis, or generated by gluconeogenesis, which shuttles into energy metabolism and anabolic biosynthetic pathways. The product of glycolysis, pyruvate, is shuttled to the pyruvate dehydrogenase complex. Then, the produced acetyl-CoA is directed to either fatty acid synthetase, or the TCA cycle protein complex. Meanwhile, ribose-5-phosphate and serine are produced from glycolytic intermediates and shunted into de novo purine biosynthesis, which is promoted by the purinosome. (B) L-Glutamine is converted to cytidine triphosphate through de novo pyrimidine biosynthesis. Used acronyms: hexokinase (HK); glucose-6-phosphate isomerase (GPI); phosphofructokinase 1 (PFK1); fructose-1,6-bisphosphatase (FBPase); triose phosphate isomerase (TIM); glyceraldehyde-3-phosphate dehydrogenase (GAPDH); phosphoglycerate kinase (PGK); phosphoglycerate mutase (PGM); phosphoenolpyruvate carboxykinase (PEPCK); pyruvate kinase (PK); pyruvate dehydrogenase (PDH); formylglycinamidine ribonucleotide synthase (FGAMS); inosine monophosphate dehydrogenase (IMPDH); adenylosuccinate synthetase (ADSS); citrate synthase (CS); acetyl-CoA oxidase (ACO); isocitrate dehydrogenase (DH); succinyl-CoA synthetase (SCS); fumarase (FM); malate dehydrogenase (MD); carbamoyl-phosphate

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synthetase, aspartate transcarbamylase, and dihydroorotase (CAD); cytidine triphosphate synthase (CTPS).

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Table 1

Evidence for Multienzyme Metabolic Complexes

metabolic pathway	in vitro evidence	fixed cell evidence (†colocalization)	live cell evidence († colocalization)	organism
glucose metabolism	mass spectrometry[26]	immunofluorescence microscopy,[25] transmission electron microscopy[24,25]	fluorescence microscopy[25]	Trypanosomatida
	co-immunoprecipitation[7,38,39] mass spectrometry[7,38,39]			plants
	co-immunoprecipitation[34]		fluorescence microscopy[33]	yeast
		Immunofluorescence microscopy[43,44]		fly
	enzyme inhibitor-binding assays[47]	immunofluorescence microscopy[46,48]†		mammal (erythro
		immunofluorescence microscopy[52,53]	fluorescence microscopy[53]†, fluorescence recovery after photobleaching,[53] intracellular fluorescence resonance energy transfer[53]†	human (cancer c
pyruvate metabolism	cryoelectron microscopy[55]			mammal
	surface plasmon resonance[56]	immunofluorescence microscopy[62,63]		human (cancer c
TCA cycle	bacterial two-hybrid analysis,[67] strep-protein interaction experiment,[67,73] in vitro reconstitution[68]			bacteria
	cross-linking and mass spectrometry,[70] diffusion analysis[71]		fluorescence recovery after photobleaching[69]	mammal
mitochondrial oxidative phosphorylation	native PAGE gel[75]			yeast
			proteomic mapping[81]	fly
	cryoelectron microscopy[76]			mammal
			proteomic mapping[6,80]	human (embryon
purine biosynthesis	Tango assay[91]		fluorescence microscopy[88,94]†, fluorescence recovery after photobleaching[92]	human (cancer c
pyrimidine biosynthesis	cryoelectron microscopy[113]	immunofluorescence microscopy[113]		bacteria
		immunofluorescence microscopy[114]	fluorescence microscopy[116]	yeast
		immunofluorescence microscopy[112]		fly
		immunofluorescence microscopy[124]		mammal
		immunofluorescence microscopy[102,114]		human (cancer c
fatty acid synthesis	X-ray crystallography[128]			bacteria
	X-ray crystallography,[129] cryoelectron microscopy[130]	immunofluorescence microscopy[132]	fluorescence microscopy[132]	yeast
	single particle cryoelectron microscopy[131]			mammal
			bioluminescence resonance energy transfer[133]†	human (cancer c
natural product biosynthesis	mass spectrometry[144]		fluorescence microscopy[144]†	bacteria
	co-immunoprecipitiation[140]	immunofluorescence microscopy[142]	fluorescence microscopy[139,140]†	fungus
amino acid metabolism	X-ray crystallography[153,155]			bacteria

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metabolic pathway	in vitro evidence	fixed cell evidence (†colocalization)	live cell evidence (†colocalization)	organism
	mass spectrometry[156,157]			mammal

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Table 2

Compartmentalization of Glycolytic Enzymes into Complexes

organism	enzymes identified to colocalize or associate in cell	subcellular localization
protists	hexokinase, PGI, PFK, aldolase, TIM, GAPDH, PGK	peroxisome
plant	hexokinase, aldolase, enolase	mitochondria
yeast	enolase, glucokinase, PGI, PFK, aldolase, TIM, GAPDH, PGM, PK	cytoplasm
Human (erythrocyte)	aldolase, GAPDH, PFK, PK	inner cell membrane
human (cancer cells)	PFK, FBPase, PKM2, PEPCK1	cytoplasm