

Freedom of assembly: metabolic enzymes come together

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ABSTRACT Many different enzymes in intermediate metabolism dynamically assemble filamentous polymers in cells, often in response to changes in physiological conditions. Most of the enzyme filaments known to date have only been observed in cells, but in a handful of cases structural and biochemical studies have revealed the mechanisms and consequences of assembly. In general, enzyme polymerization functions as a mechanism to allosterically tune enzyme kinetics, and it may play a physiological role in integrating metabolic signaling. Here, we highlight some principles of metabolic filaments by focusing on two well-studied examples in nucleotide biosynthesis pathways—inosine-5'-monophosphate (IMP) dehydrogenase and cytosine triphosphate (CTP) synthase.

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INTRODUCTION

Classically, intermediate metabolism has been viewed as a kind of soup of enzymes and substrates, partially organized by sequestration into membrane-bound compartments and limited primarily by diffusion within those compartments. More recently, however, a significant level of physical organization of intermediate metabolism has been uncovered, where dynamic reorganization of enzymes into discreet cellular structures is linked to changes in metabolic conditions. These structures include multienzyme aggregates that colocalize different enzymes in a pathway, and filamentous polymers that form by self-assembly of a single enzyme (Chitrakar *et al.*, 2017; Jin *et al.*, 2017).

Several dozen different metabolic enzymes are now known to form filamentous polymers in cells (for a comprehensive recent review, see Park and Horton, 2019). Among the best described of these metabolic filaments are IMP dehydrogenase (IMPDH) and CTP synthase (CTPS), key regulatory enzymes in the purine and pyrimidine nucleotide biosynthesis pathways, respectively. Here, we focus on these two examples to highlight common aspects of metabolic filament structure, evolution, regulation, and molecular mechanisms.

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Abbreviations used: ATP, adenosine triphosphate; CTP, cytosine triphosphate; CTPS, CTP synthase; GTP, guanosine triphosphate; IMP, inosine monophosphate; IMPDH, IMP dehydrogenase; WT, wild type.

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Evolution of assembly

How metabolic enzyme polymerization arose during evolution is not well understood. However, theoretical and experimental models suggest that single amino acid alterations can be sufficient to drive otherwise diffuse enzymes to assemble (Garcia-Seisdedos *et al.*, 2017). This may be especially true for metabolic enzymes, which are frequently homo-oligomeric proteins, and therefore the effect of individual mutations can be amplified by their presence in each subunit (Figure 1). Indeed, many proteins, and particularly metabolic enzymes, have been found to form large assemblies in proteomic and imaging-based screens in yeast (Noree *et al.*, 2010, 2019; O’Connell *et al.*, 2014; Shen *et al.*, 2016).

The evolution of polymerization in CTPS and IMPDH each followed a unique trajectory, but illustrate principals that may be relevant to other polymerizing enzymes. Eukaryotic and prokaryotic CTPS form a conserved tetrameric structure, but adopt very different filamentous forms mediated by different assembly contacts (Figure 2, A and B). In bacteria, filaments form from interlocking tetramers, while a unique 10 amino acid insertion mediates stacking of eukaryotic CTPS tetramers into filaments (Barry *et al.*, 2014; Lynch *et al.*, 2017; Lynch and Kollman, 2020). The insertion can be found in some protist and plant CTPS enzymes, suggesting that this form of CTPS polymer may have evolved ~1.5 billion years ago. The evolution of this insert may have driven a transition in CTPS polymer morphology, with the corresponding functional consequences outlined below. Alternatively, eukaryotic and prokaryotic polymer forms may have evolved independently from a nonpolymerizing ancestor, which again would be consistent with the idea that polymerization of homo-oligomeric proteins can be readily achieved with a small number of mutations.

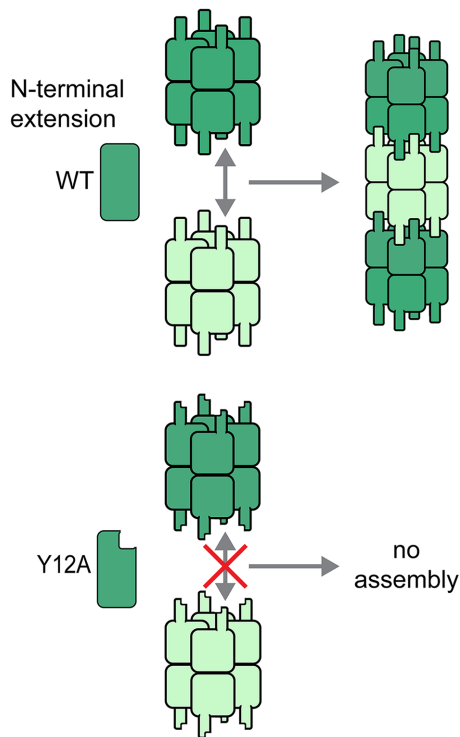


FIGURE 1: A single amino acid change in the IMPDH N-terminal extension disrupts assembly of IMPDH octamers. Intermolecular interactions between IMPDH protomers are amplified eightfold through reciprocal interactions across the polymerization interface between octamers. The avidity effects of such multivalent interactions may facilitate evolution of polymers of oligomeric proteins. Conversely, mutation of just one amino acid, tyrosine 12 to alanine, in the N-terminal extension, completely prevents octamer polymerization.

Polymerization of IMPDH appears to have evolved more recently than for CTPS. An N-terminal sequence extension, conserved in most metazoan IMPDH enzymes, plays a key role in mediating polymerization of octamers by a mechanism related to “runaway domain coupling” (Figure 2, C and D; McPartland *et al.*, 2018). Recent high-resolution cryo-EM structures have shown that the flexible N-terminus extends from the enzyme core to make ordered interactions across an assembly interface, and a single alanine substitution mutation in tyrosine 12 prevents the formation of IMPDH filaments (Anthony *et al.*, 2017; Fernández-Justel *et al.*, 2019; Johnson and Kollman, 2020). At each octamer–octamer interface, this reciprocal interaction is seen in all eight adjacent monomers, dramatically increasing the valency and buried surface area of the interaction, potentially explaining the key role of this residue. IMPDH filaments have thus far only been demonstrated in mammalian cells and despite extensive analysis are not observed in *Drosophila*. Consistent with this, tyrosine 12 and the surrounding amino acid sequence are not conserved in *Diptera* but are present in some species, such as *Trichoplax adhaerens*, that are more distantly related to mammals. This suggests that IMPDH assembly may have evolved during the development of multicellularity but has been lost in certain animal lineages. Consistent with their unique evolutionary trajectories, different regulatory mechanisms modulate the assembly of CTPS and IMPDH, as described next.

Regulation of assembly

In metazoans, assembly of both IMPDH and CTPS is associated with cellular conditions in which nucleotide demand exceeds supply. For

example, in *Drosophila* ovary or salivary gland, CTPS assembly correlates with developmental periods in which cells undergo endoreplication (Liu, 2010), a process that dramatically increases nucleotide demand. CTPS filaments have also been observed in rapidly dividing cancer cells in culture (Chang *et al.*, 2017), and mammalian T-cells transiently assemble IMPDH into filaments in response to immunological activation before cell proliferation (Calise *et al.*, 2018; Duong-Ly *et al.*, 2018). It remains to be seen whether assembly in these contexts is simply a response to changes in nucleotide abundance relative to demand or whether it is part of a programmed change in cell state.

Filament assembly is also observed when nucleotide supply is limited, such as in cells treated with pharmacological inhibitors of nucleotide biosynthetic enzymes (reviewed in Schiavon *et al.*, 2018). Conversely, increasing nucleotide levels by providing them exogenously triggers filament dispersion (Gunter *et al.*, 2008; Calise *et al.*, 2014, 2016; Duong-Ly *et al.*, 2018). These findings are consistent with emerging models for assembly as a means of regulating nucleotide homeostasis (Barry *et al.*, 2014; Gou *et al.*, 2014; Noree *et al.*, 2014; Calise *et al.*, 2016; Anthony *et al.*, 2017; Lynch *et al.*, 2017; Fernández-Justel *et al.*, 2019).

Elevating IMPDH or CTPS protein levels in cells also promotes their assembly (Ingerson-Mahar *et al.*, 2010; Keppeke *et al.*, 2018; Li *et al.*, 2018; Wu and Liu, 2019). IMPDH protein is dramatically up-regulated in primary T-cells following T-cell receptor (TCR) activation and is associated with IMPDH assembly. However, increased IMPDH expression is not sufficient for assembly, which also depends on signaling by NFAT and mTOR (Duong-Ly *et al.*, 2018). mTOR has also been implicated in CTPS filament formation in *Drosophila* (Aughey *et al.*, 2014; Sun and Liu, 2019). In *Drosophila* ovaries, CTPS filament assembly is regulated by Myc and activated cdc42-associated kinase (Ack; Strohlic *et al.*, 2014; Aughey *et al.*, 2016). It remains unknown how these pathways impact assembly but posttranslational modifications of the enzymes themselves could be involved. CTPS assembly in the *Drosophila* ovary is regulated by ubiquitination associated with the Cbl ubiquitin ligase (Wang *et al.*, 2015). In cultured cells under nutrient stress, CTPS filament assembly is regulated by methylation controlled by histidine and the folate cycle (Lin *et al.*, 2018). However, as of yet there does not appear to be a clear connection between these posttranslational modifications and known signaling pathways associated with filament assembly.

In addition to regulation by signaling pathways, small molecules including substrates, products, and allosteric regulators can influence metabolic enzyme polymerization. ATP and GTP both allosterically regulate filament assembly and activity of IMPDH (Anthony *et al.*, 2017; Buey *et al.*, 2017; Fernández-Justel *et al.*, 2019) and GTP allosterically regulates CTPS activity (Habrian *et al.*, 2016). Likewise, the presence or absence of their substrates strongly influences CTPS and IMPDH polymer assembly *in vitro* (Anthony *et al.*, 2017; Lynch *et al.*, 2017). Interestingly, in cultured cells, CTPS and IMPDH can assemble into distinct structures or can assemble together, depending on the method of induction (Carcamo *et al.*, 2011; Chang *et al.*, 2015, 2018). This suggests the intriguing possibility that coassembly could mediate coordination between purine and pyrimidine biosynthetic pathways. Consistent with this, pharmacological inhibitors of CTPS promote the assembly of IMPDH filaments (Carcamo *et al.*, 2011; Chang *et al.*, 2015, 2018).

These examples highlight the complexity of regulation of metabolic enzyme assembly. Future work must begin to connect these different levels of regulation to form a complete picture of how cells sense their metabolic state and initiate enzyme polymerization.

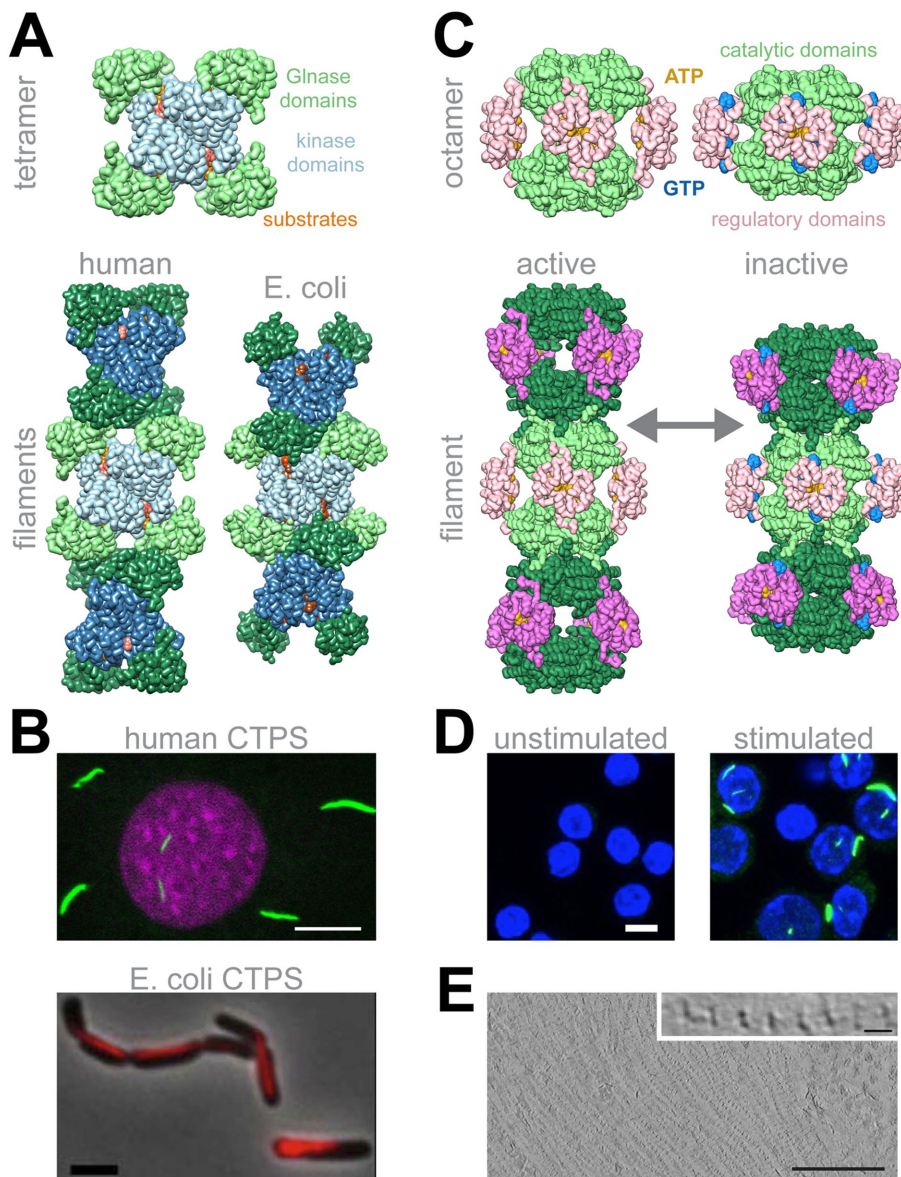


FIGURE 2: Structures of CTPS and IMPDH filaments. (A) CTPS structures. CTPS monomers each have two catalytic domains (blue and green), and assemble into tetramers. Tetramers can dynamically assemble filaments with different geometries. A unique eukaryotic insert mediates assembly contacts of human CTPS, while *Escherichia coli* CTPS tetramers form more extensive, interlocking interactions. (B) Cellular polymerization of CTPS is broadly conserved (adapted from Barry *et al.* 2014; Gou *et al.*, 2014). (C) IMPDH structures. IMPDH is an octamer, with each protomer consisting of catalytic (green) and regulatory (pink) domains. ATP binding in the regulatory domains stabilizes octamers, and GTP binding promotes a conformational change in that, in the context of the octamer and in the filament, results in a compressed, inactive conformation. (D) IMPDH polymers are observed to form in mouse lymphocytes upon TCR stimulation (adapted from Duong-Ly *et al.* 2018). (E) Cryo-tomography of HEp-2 cells shows IMPDH forming extensive bundles with spacing consistent with the spacing observed in *in vitro* reconstituted single filaments (adapted from Juda *et al.* 2014). Scale bars: B (top) = 10 μ m, B (bottom) = 3 μ m, D = 5 μ m, E = 200 nm, inset in E = 10 nm.

Assembly is an allosteric regulator

Although there are only a handful of examples of studies on the biochemical effects of polymerization, it appears that in most cases filament assembly directly tunes enzyme kinetics. Assembly-based allosteric tuning can take very different forms, including decreasing or increasing specific activity, altering the response to other alloste-

ric regulators, or enhancing the cooperativity with which a population of enzymes transitions between activity states (Figure 3).

Tetrameric CTPS has a conserved conformational equilibrium, with cooperative transitions between active and inactive conformations resulting from relative motions of the two catalytic domains in each protomer (Lynch *et al.*, 2017). The inactive conformation is promoted by CTP binding, in a form of feedback inhibition. Filaments provide an additional layer of allosteric control on top of this conformational equilibrium, although these effects vary between domains of life. For example, in bacteria, filament assembly interactions sterically lock the CTPS tetramer into the inactive conformation (Figure 2A; Barry *et al.*, 2014; Lynch *et al.*, 2017). This suggests that for bacterial CTPS, polymerization is a mechanism to maintain homeostasis by rapidly shutting down activity in response to elevated CTP levels. A similar mechanism was recently described for filaments of budding yeast glucokinase 1, suggesting that polymerization can act as a “molecular surge protector” to prevent deleterious metabolic effects of excess flux through the initial phase of glycolysis (Stoddard *et al.*, 2020).

Humans have two CTPS isoforms, CTPS1 and CTPS2, that both form filaments with interaction interfaces that are completely different from the bacterial interface (Figure 2A). The biochemical consequences of polymerization vary between the two human isoforms. CTPS1 is much more active in filaments, and disassembles upon inactivation (Lynch *et al.*, 2017). CTPS2, thought to be the more “housekeeping” isoform, is able to transition between active and inactive conformations while remaining assembled; in this case, coupling conformational changes between many CTPS tetramers increases cooperativity of the structural transition between active and inactive states (Lynch and Kollman, 2020). These different regulatory outcomes likely reflect the different cellular roles of the two isoforms: shifting CTPS1 into polymers would increase flux through the enzyme to produce the higher nucleotide levels required during proliferation, while enhanced cooperativity in CTPS2 filaments provides a mechanism for ultrasensitive, switch-like control of CTP synthesis during more homeostatic conditions. Like CTPS1, other metabolic enzymes appear to be stabilized in active conformations in filaments (Webb

et al., 2017; Kim *et al.*, 2019). And like CTPS2, at least one other enzyme filament, acetyl-CoA carboxylase, can form filaments in active or inactive states depending on what allosteric ligands or regulatory partners are bound (Hunkeler *et al.*, 2018).

Like CTPS2, IMPDH filaments can accommodate active or inactive conformations (Anthony *et al.*, 2017), although in this case

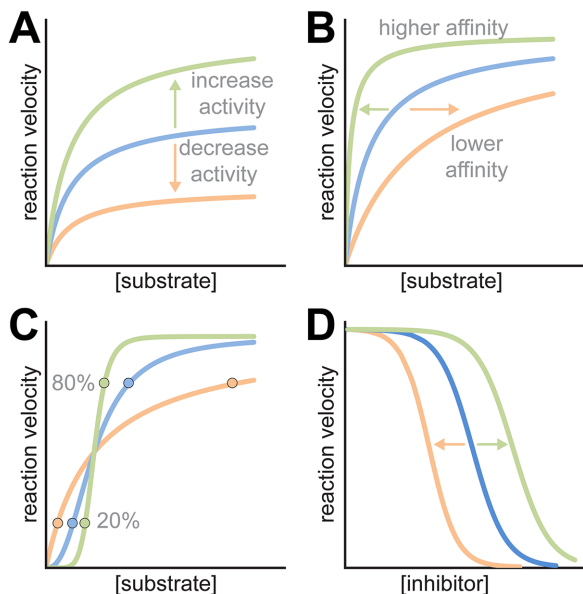


FIGURE 3: Potential effects of polymerization on enzyme kinetics. Hypothetical activity of free enzymes (blue) is compared with possible activity in filaments (green and orange). (A) Filaments can affect the catalytic rate constant, either increasing or decreasing activity across all concentrations of substrate. (B) Filaments might affect affinity for substrate (the apparent K_m), resulting in different activity levels at low substrate concentrations. (C) Filaments can influence the cooperativity of reactions. Circles show the 20 and 80% activity levels, highlighting the steepening of the activity curve with increasing cooperativity. (D) Filaments can shift the sensitivity to allosteric effectors, here illustrated as an inhibitor.

assembly does not have a direct effect on specific activity and does not enhance cooperativity (Figure 2C). Instead, IMPDH filament assembly is sensitive to the levels of both substrate and downstream product (GTP) pools. In the presence of substrate, filament assembly interactions stabilize an enzyme conformation that resists allosteric inhibition by the downstream product GTP (Johnson and Kollman, 2020). This allows IMPDH to retain activity in the presence of otherwise inhibitory GTP concentrations, consistent with the high nucleotide demands where IMPDH assembly is observed, such as lymphocyte activation (Calise *et al.*, 2018; Duong-Ly *et al.*, 2018).

IMPDH filaments have also been shown to form large, lateral bundles in cells; these bundles have a periodicity consistent with the spacing between octamers in *in vitro* reconstituted single filaments (Figure 2E; Juda *et al.*, 2014). Given the large size of most cellular metabolic filaments, we anticipate that this kind of lateral bundling of single filaments may be a common form of higher order association, although the functional importance of bundles has not yet been explored for any enzyme filament.

Why filaments?

It may seem surprising that filament assembly has evolved as a general mechanism for tuning enzyme kinetics; after all, there are limitless examples in metabolism of allosteric control being achieved through smaller, defined oligomers. One simple explanation may be that polymer assembly is relatively easy to evolve with a small number of mutations, as suggested above (Garcia-Seisdedos *et al.*, 2017). By this logic, filament assembly is a straightforward way to introduce an additional layer of regulation on top of existing allostery.

Nevertheless, it seems peculiarly baroque to build micron-scale cellular structures simply to regulate the activity of a single enzyme. One intriguing possibility is that large, ordered, multivalent structures like metabolic filaments may serve as platforms for signaling the state of cellular metabolism. There are hints from cell biological studies that this might be the case. For example, coassembly of IMPDH and CTPS filaments suggests the possibility that biosynthetic activity of these two separate but interrelated pathways may be coregulated through physical interaction, whether direct or mediated by an as yet unidentified scaffold. Even more intriguing, other signaling molecules colocalize with these filaments (Strochlic *et al.*, 2014; Hayward *et al.*, 2019), supporting the idea the filaments may be coordinating multiple activities in response to changing metabolic conditions.

Outlook

While many enzyme filaments have been observed in cells, only a few have been characterized at the level of structure and biochemical function. Given the diversity of structural and regulatory outcomes of the few cases where these details are known, it seems likely that other novel mechanisms remain to be discovered.

One pressing challenge is to uncover the physiological role of the various metabolic filaments. *In vitro* studies of filament structures, assembly mechanisms, and biochemical regulation are providing context and tools for studying the role of metabolic filaments in cells and tissues. Understanding how filaments specifically alter metabolite levels and/or flux through the associated pathways under varying physiological conditions is an important next step in understanding their biological function. Identifying whether other cellular factors specifically recognize and interact with metabolic filaments is another important step in understanding how they communicate with other cellular components.

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