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MicroCommentary

Multifunctional enzymes from reduced genomes – model proteins for simple primordial metabolism?

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Summary

Billions of years of evolution have yielded today's complex metabolic networks driven by efficient and highly specialized enzymes. In contrast, the metabolism of the earliest cellular life forms was likely much simpler with only a few enzymes of comparatively low activity. It has been speculated that these early enzymes had low specificities and in turn were able to perform multiple functions. In this issue of Molecular Microbiology, Ferla *et al*. describe examples of enzymes that catalyze chemically distinct reactions while using the same active site. Most importantly, the authors demonstrated that the comparatively weak activities of these multifunctional enzymes are each physiologically relevant. These findings contrast with simply promiscuous enzyme activities, which have been described numerous times but are not physiologically relevant. Ferla *et al*. elegantly combined initial bioinformatics searches for enzyme candidates with sound kinetic measurements, evolutionary considerations and even structural discussions. The phenomenon of multifunctionality appears to be a mechanism for bacteria with reduced genomes to compensate for their lack of certain enzymes. In the broader context of evolution, these organisms could be considered living model systems to study features of long-extinct early cellular life.

The classic view of enzymes pictures biocatalysts that accelerate the rate of a reaction by many orders of magnitude and act with a high level of specificity. The first part of this traditional view has already been rectified by the seminal meta-analysis of published biochemical data on thousands of enzymes, which found that the average enzyme is in fact much less active than the often-cited textbook examples (Bar-Even *et al*., 2011). Furthermore, while many enzymes exhibit high specificity with respect to the catalyzed reaction and the nature of the substrate, it has now been widely appreciated that numerous enzymes show catalytic or substrate promiscuity – they can also catalyze different chemical reactions or accept a range of diverse substrates (Khersonsky and Tawfik, 2010). This secondary activity is usually orders of magnitude lower and, by definition, not physiologically relevant (Copley, 2015). Nevertheless, promiscuity has been appreciated as an important starting point for the evolution of distinctly new enzymes (Copley, 2014; Khersonsky *et al*., 2006). The steady stream of newly identified promiscuous activities lets one wonder whether enzyme promiscuity might be more the rule than the exception. There are likely many more promiscuous activities to be found, especially when including low catalytic activities in the search.

The starting point for the new study by Ferla *et al*. was the previous identification by the Patrick lab of a promiscuous alanine racemase (ALR) activity in the cystathionine β-lyase enzyme (CBL) from *Escherichia coli* (Soo *et al*., 2016). Notably, the canonical ALR enzyme and the CBL enzyme are entirely unrelated. ALR produces D-alanine which is crucial for peptidoglycan biosynthesis, while CBL is a key enzyme in the methionine pathway and yields homocysteine. The only aspect the two enzymes have in common is the use of the cofactor pyridoxal 5′-phosphate (PLP). At the outset of this new study, Ferla *et al*. asked whether there might have been a bifunctional CBL/ALR ancestor of the modern CBL and only later the ALR function was overtaken by a new ALR specialist enzyme. If that was the case, they wondered if such multifunctional CBL enzymes could still be found in extant bacteria. Ferla *et al*., therefore, devised a clever search strategy to address this question.

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Identifying multifunctional enzymes – *in silico* search and *in vivo* confirmation

To identify candidate CBL variants that have a physiologically relevant ALR activity, a bioinformatics search was performed on >1000 sequenced bacterial genomes. The search criteria were the presence of a *metC* gene (encoding CBL), the concomitant absence of an *alr* gene, and a requirement for peptidoglycan synthesis. Interestingly, the three taxa that satisfied all search criteria all have reduced genomes (the alphaproteobacterial orders *Pelagibacterales* and *Rickettsiales*; and the genus *Thermotoga*). To test the respective enzyme variants (*Pu*CBL, *w*MelCBL, *Tm*CBL) encoded by the three identified *metC* genes for CBL and ALR activities, complementation assays were performed. Each of the three *metC* variants were expressed on a plasmid in the *E. coli* Δ*metC* strain from the Keio collection (Baba *et al*., 2006) and the D-alanine auxotroph *E. coli* MB2795 (Δ*alr* Δ*dadX*) (Soo *et al*., 2016). All three variants were found to have both the CBL and ALR activity as they rescued growth in the methionine and the D-alanine auxotroph. The ALR-based rescue was as rapid as the positive control with the wild type *E. coli* ALR enzyme, while the methionine complementation was substantially slower. All three *metC* variants were also heterologously expressed and purified for the determination of the kinetic parameters for both their ALR and CBL activities.

A surprise finding of the third kind

Ferla *et al*. noticed that the three genomes chosen after the bioinformatics search were also lacking glutamate racemase (GLR). The GLR enzyme is needed to synthesize D-glutamate, another crucial ingredient of peptidoglycan polymer (Schleifer and Kandler, 1972). Indeed, two of the three identified CBL enzymes were shown to also possess the GLR activity. This finding is particularly remarkable because all characterized GLR enzymes do not use a PLP cofactor and are unrelated to CBL and ALR in sequence, structure and mechanism.

Probing the physiological relevance of the CBL variant from *Thermotoga maritima*

The purified *Tm*CBL enzyme variant from *T. maritima* was shown to possess *in vitro* activities for the CBL, ALR and GLR reactions. But detailed biochemical investigations on the methionine biosynthesis in *T. maritima* showed that the CBL activity of *Tm*CBL appears to not be physiological because the canonical CBL substrate cystathionine is never synthesized in this organism. Yet, after further careful studies, Ferla *et al*. came to the conclusion that both the ALR and GLR activities of *Tm*CBL are physiological. It is difficult to prove beyond doubt that a given enzyme with a specific activity is indeed the only enzyme that catalyzes a particular reaction. Ferla *et al*. ruled out any reasonable alternative by testing six of the candidates individually. The likely ability of many enzymes to possess secondary activities is clearly complicating such studies. Until genetic tools are developed for non-model organisms such as *T. maritima*, there will always be some uncertainty left.

Considering evolutionary and structural relationships

The comparison of phylogenetic trees of representative CBL enzymes with those of concatenated 16S and 23S rRNA sequences suggested that multifunctional CBL enzymes have frequently moved between species through horizontal gene transfer. Such a transfer of a bifunctional ALR/GLR enzyme could in turn have enabled some organisms to dispose of their canonical ALR and GLR genes, especially if under selection pressure to reduce their genome. For the three identified PuCBL, wMelCBL and TmCBL enzymes, structural homology modeling confirmed high similarity. Ferla *et al*. were even able to relate activity differences between the variants to mutations in crucial positions in the respective active sites.

Implications – connecting reduced genomes with primordial metabolism and synthetic biology

The original hypothesis by Ferla *et al*. suggested that modern CBL may have evolved from a bifunctional CBL/ALR ancestor and later, unrelated ALR specialists took over. While a sound hypothesis, this was not confirmed, but instead the experimental work led to even more interesting findings. Great research projects often turn out this way and thereby keep us scientists more excited at each turn. To date, only very few examples for bifunctional enzymes beyond the study by Ferla *et al*. have been reported (Adams *et al*., 2014; Barona-Gómez and Hodgson, 2003; Say and Fuchs, 2010). Several of those are also from organisms with relatively small genomes. Interestingly, the most abundant organisms on earth are bacteria with reduced genomes.

As the primordial metabolism in early cellular life is thought to have been facilitated by simple multifunctional, yet likely slow enzymes (Jensen, 1976; Yčas, 1974), bacteria with reduced genomes could be considered living model systems to demonstrate and study this principle (Fig. 1). Furthermore, the design of a functioning

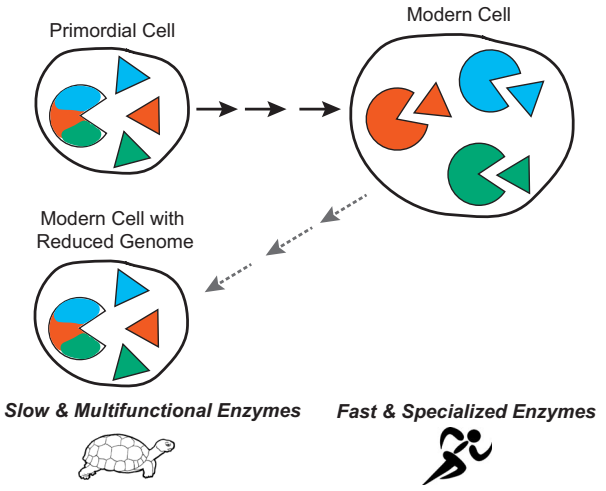

Fig. 1. Schematic representation of the evolution from slow multifunctional enzymes that were likely powering primitive early cells to highly active and specialized enzymes that drive modern cells. Under conditions that favor genome reduction, the evolution 'back' to multifunctional enzymes can compensate for the lack of certain enzymes in modern bacterial cells as shown by Ferla *et al.* (2017) in this issue of Molecular Microbiology. These multifunctional but relatively slow enzymes can be used as proxies to study primordial-like enzyme variants.

organism from a minimal set of components is an important aim of synthetic biology (Caschera and Noireaux, 2014). Toward that goal, a minimal set of biochemical activities crucial for life (or universal gene set of life) has been discussed (Harris *et al.*, 2003; Juhas *et al.*, 2011). Suitable multifunctional enzymes will greatly facilitate the construction of such a synthetic minimal cell. By dissecting and simplifying the complicated workings of the cell, this line of research will enable a better understanding of early living systems, but also metabolic networks in general.

While the best-known textbook examples of enzymes are undoubtedly impressive as they accelerate reactions to the diffusion rate limit and with highest specificity, the world – and especially microbial life – might be ruled by enzymes that are rather slow, but have more than one function.

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