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The Role of Inhibition in Enzyme Evolution

Crosstalk

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Most people believe that new enzymes evolve from existing enzymes because of the conservation of amino acid sequence and tertiary structures in enzymes with different functions and the low probability that such similarities could evolve independently. However, the process by which this divergence occurs is still debatable. A reasonable proposal for this process is the duplication of a gene encoding an existing enzyme, accumulation of mutations in the gene duplicate(s) with consequential functional divergence, followed by selection for an enzyme with a new function [1]. To substantiate this proposal, several areas must be addressed. Which proteins serve as templates? Under what conditions would this series of events occur? What selective advantage does a new enzyme confer upon the host?

Much discussion has focused on templates, with most suggesting that enzymes that catalyze alternate reactions or with the potential to do so with a few mutations being the most likely candidates. Jensen suggested that enzymes that catalyze a secondary reaction might serve as templates for the evolution of enzymes that specialize in catalyzing the secondary reaction—"substrate ambiguity" [2]. Building upon this idea, O'Brien and Herschlag added that the secondary reaction need not be related to the primary reaction—"catalytic promiscuity" [3]. Gerlt and Babbitt point out that many structurally related enzymes catalyze different reactions with identical half reactions, and therefore suggest that, in many cases, mechanism is conserved while binding capacity evolves [4].

The question of suitable conditions for divergence has received less attention. Gene duplications have been documented to occur in response to extended growth at high temperatures, exposure to toxins, and from growth in the absence of essential nutrients [5, 6]. Duplications offer two advantages to a cell under such conditions. One, duplications increase gene concentration and consequently the enzyme concentration, which is a crude form of upregulation. Two, multiple gene copies provide a buffering effect against deleterious mutations, while mutations that cause functional divergence accumulate.

Regarding selective advantage, it is generally assumed that the benefit to the host from having a new enzyme is the provision of additional nutrients that enter existing metabolic pathways. Under growth-limiting conditions, these extra metabolites allow the host to reproduce faster than its neighbors, and the new enzyme becomes fixed in the population.

One facet of protein function that has not been widely considered in the context of general protein evolution is inhibition. In this article, we refer to inhibitors simply as any compound that competes with the primary substrate for the active site of an enzyme. The regulation of enzyme activity via feedback inhibition will not be discussed here. It is well known that compounds that bind to the active site of an enzyme interfere with the primary function of the protein. It is recognized that such reduction in the primary function can reduce the growth rate of the host if the primary reaction is growth-rate limiting. Therefore, it is logical that an organism that develops the means of reducing the degree of inhibition will have a growth advantage over its peers. The means can take the form of preventing the inhibitor from accessing the enzyme via physical processes like compartmentalization or active transport gateways through membranes. Another method is to express an enzyme only in the presence of high concentrations of its primary substrate, which outcompetes the inhibiting compounds (gene regulation).

However, in the context of divergent evolution, an enzyme variant with lower affinity for an inhibitor or the ability to decompose an inhibitor will generate a selective advantage under conditions where the growth-rate-limiting function of the wild-type enzyme is being inhibited. Studies on the mechanisms by which organisms develop resistance to toxic compounds like antibiotics, pesticides, and anticancer drugs are evidence that this process has occurred. In some instances, the evolved enzyme is an isozyme of its inhibited progenitor, retaining the primary function but not subject to inhibition by the toxin. For example, dihydrofolate reductase forms with low affinity for the inhibitor methotrexate, yet normal reductase activity evolved in murine cancer cells exposed to this anticancer drug [7].

In other cases, the evolved enzyme is essentially a new enzyme with activity toward the compound that inhibits the primary activity of its progenitor. An example of this has been documented in blowfly populations exposed to the organophosphate pesticide diazinon. Organophosphates inhibit the primary function of carboxylesterases, which has the effect of preventing normal nerve function and causes death to the insect. The active site serine readily attacks the organophosphate, but the resulting phosphoester bond is very slowly hydrolyzed. In a sense, these enzymes are primarily carboxylesterases with an organophosphate hydrolase alternate activity. In resistant populations of blowfly, a single amino acid change in a carboxylesterase greatly enhances the organophosphate hydrolase activity while ameliorating the carboxylesterase activity [8]. It is important to note that the hydrolysis products of diazinon offer little if any selective advantage to the host as me-

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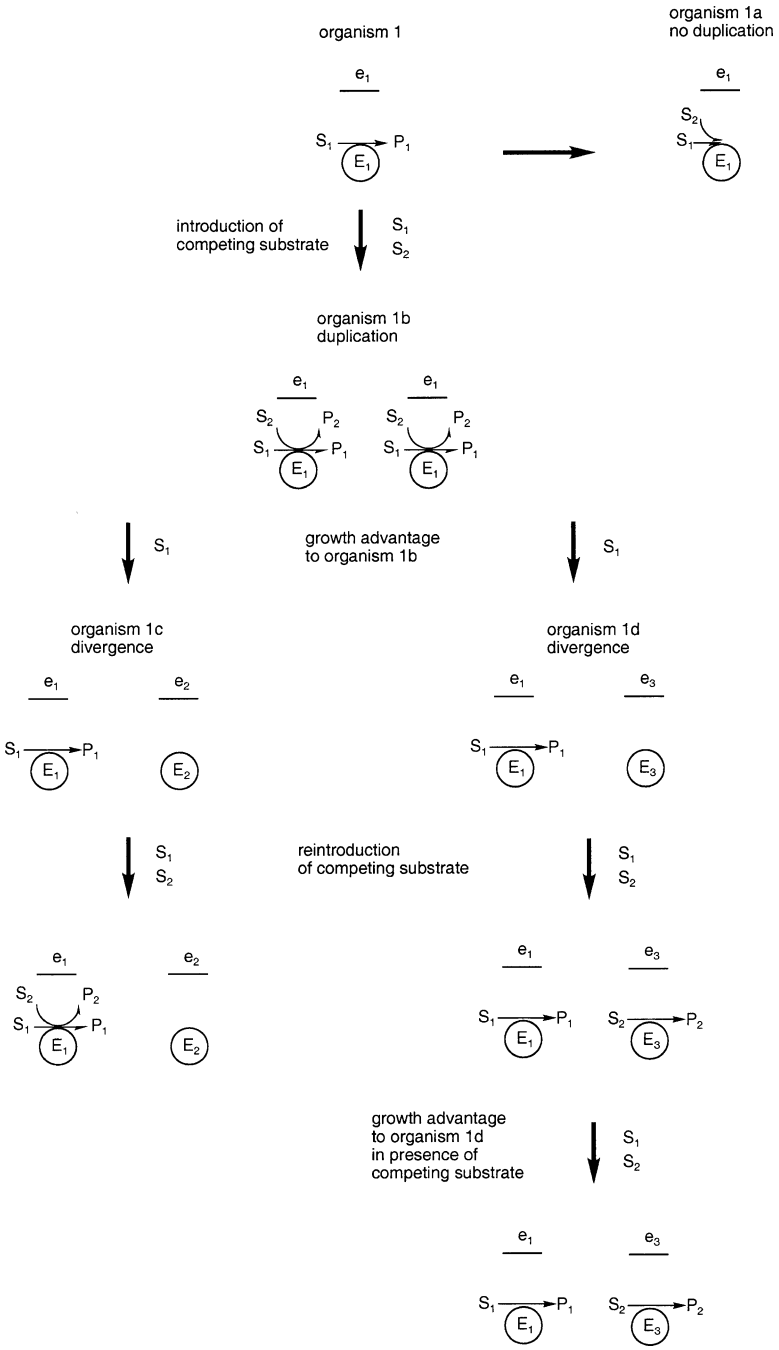

Figure 1. Diagram Depicting Hypothetical Flow of Events for Divergent Evolution from an Inhibited Enzyme

S_1, primary substrate; P_1, product(s) of primary substrate; S_2, secondary/competing substrate; P_2, product(s) of secondary/competing substrate; e_1, gene ecoding promiscuous enzyme, E_1; E_1, wild-type; $E_{2\text{-}5}$, mutants; E_2, primary and secondary activities KO; E_3, new enzyme in which wild-type secondary activity has become primary activity. Half arrows indicate reduced activity; full arrows indicate full activity. The deletion of organisms 1a, 1b, and 1c from the figure illustrates a relative reduction in numbers in the population, not a removal. Organism 1d is the dominant member of the population.

tabolites. Essentially, the evolution of the organophosphate hydrolase generated a selective advantage to the host organism in response to the inhibition of an enzyme-inhibiting and growth-rate-inhibiting compound.

The evolution of these resistance enzymes followed a pattern resembling that described earlier, a generalized form of which is depicted in Figure 1. In the evolution of dihydrofolate reductase isozymes, initial resistance developed in cells in which duplication of the gene encoding the inhibited wild-type enzyme occurred. The increased cellular concentration of the inhibited enzyme increased the net activity and provided a growth advantage. Further selective advantage was gained from the evolution of inhibitor-resistant isozymes from among the duplicated population. While this series of events has not been replicated in the lab for blowflies, there is evidence of extensive gene duplication of carboxylesterase genes in insect populations exposed to organophosphates in the wild [9]. Like with dihydrofolate reductase in murine cells, it was the gene encoding the inhibited enzyme that was the progenitor for the new gene encoding the new enzyme.

Organophosphate pesticides are usually thought of as inhibitors and not as alternate substrates for carboxylesterases because of their overall effect on the reproduction of the host. It is worth considering whether com-

pounds normally thought of as alternate substrates can act as inhibitors of the primary function of an enzyme. Here, as long as the inhibition is at the active site, then the more important factor is the degree of inhibition, not the type. Alternate substrates inhibit the turnover of the primary substrate by an enzyme in vitro (e.g., diisopropylfluorophosphate hydrolysis competitively inhibits paraoxon hydrolysis by phosphotriesterase [10]). It is reasonable to assume that if the paraoxon hydrolysis activity of phosphotriesterase were growth-rate limiting to an organism, then the addition of inhibitory concentrations of diisopropylfluorophosphate would slow growth. A recent study in our lab found that the growth of *Escherichia coli* engineered to grow using dimethyl phosphate as a sole phosphorus source was inhibited by the addition of paraoxon to the growth media [11]. In vitro assays found that the enzyme that catalyzed the hydrolysis of dimethyl phosphate, a broad specificity phosphohydrolase from *Enterobacter aerogenes*, also had activity toward paraoxon but not toward noninhibitory compounds like parathion at the same concentration.

There are two ways in which enzyme inhibition may complement the previously mentioned ideas on enzyme evolution. First, the presence of an inhibitor actually selects for the duplication of a gene encoding an enzyme to which it already has affinity. When a new compound enters a cell, any expressed enzyme to which the compound has affinity is a potential template for divergent evolution because inhibition itself is enough to select for duplication events. Naturally, not all compounds that have affinity for an enzyme are potential substrates. However, a compound that binds to the active site of an enzyme is halfway toward being acted upon. And one of the first advantageous responses to inhibition is duplication, which is almost always required for functional divergence. If the effects of inhibition are ignored, the presence of an alternate substrate cannot select for duplication of a suitable gene (or any gene, for that matter). Therefore, duplication of genes encoding an enzyme with a low, alternate activity is independent of the alternate substrate. Secondly, the evolution of a new enzyme from an inhibited enzyme provides a selective advantage to the cell whether or not the product is a source of nutrition. The inhibited enzyme is no longer inhibited and can resume normal function. If the product of the new enzyme can enter an existing metabolic pathway, then the benefit to the cell is at both levels. This point may also be of some relevance to discussion on the related topic of the evolution of metabolic pathways ([12] for overview). The fundamental problem is that there is no advantage to the host to have an enzyme that is not part of a metabolic pathway. Therefore, multicomponent pathways must evolve in the right order so that a useful metabolite is always generated. The evolution of a new enzyme that removes an inhibitor generates a selective advantage in one step, irrespective of the fate of the products. Once enough components are present, rudimentary pathways may then be functionally patched together, providing the host with useful building blocks.

It is important to note that these described cases of duplication and divergence have been in response to the periodic application of conditions that reduced the flux along an essential metabolic pathway. The growth phase between application of toxins like pesticides allows for the accumulation of mutations in the duplicate genes with no harmful effects on the host, as the full primary function of the duplicates is only required in the presence of the toxin. Of course, the periodic application of toxins can be broadened in concept to the periodic application of any selection pressure for which divergent evolution from gene duplicates is advantageous; the evolution of a ribitol dehydrogenase with greater activity toward xylitol being a good case [6].

If inhibition from competing substrates is a factor in new enzyme evolution, then, in theory, inhibition may also influence the evolution of other biomolecules, both catalytic and noncatalytic. While the role of inhibition in the evolution of new enzymes is no doubt just one that acts in concert with other selection factors, this concept may explain the mechanism for the generation and selection of divergent enzymes in many cases.

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References

1. Lewis, E.B. (1951). Pseudoallelism and gene evolution. Cold Spring Harb. Symp. Quant. Biol. *16*, 159–174.
2. Jensen, R.A. (1976). Enzyme recruitment in evolution of new function. Annu. Rev. Microbiol. *30*, 409–425.
3. O'Brien, P.J., and Herschlag, D. (1999). Catalytic promiscuity and the evolution of new enzymatic activities. Chem. Biol. *6*, R91–R105.
4. Gerlt, J.A., and Babbitt, P.C. (1998). Mechanistically diverse enzyme superfamilies: the importance of chemistry in the evolution of catalysis. Curr. Opin. Chem. Biol. *2*, 607–612.
5. Riehle, M.M., Bennett, A.F., and Long, A.D. (2001). Genetic architecture of thermal adaptation in Escherichia coli. Proc. Natl. Acad. Sci. USA *98*, 525–530.
6. Rigby, P.W., Burleigh, B.D., Jr., and Hartley, B.S. (1974). Gene duplication in experimental enzyme evolution. Nature *251*, 200–204.
7. Haber, D.A., Beverley, S.M., Kiely, M.L., and Schimke, R.T. (1981). Properties of an altered dihydrofolate reductase encoded by amplified genes in cultured mouse fibroblasts. J. Biol. Chem. *256*, 9501–9510.
8. Newcomb, R.D., Campbell, P.M., Ollis, D.L., Cheah, E., Russell, R.J., and Oakeshott, J.G. (1997). A single amino acid substitution converts a carboxylesterase to an organophosphorus hydrolase and confers insecticide resistance on a blowfly. Proc. Natl. Acad. Sci. USA *94*, 7464–7468.
9. Devonshire, A.L., and Field, L.M. (1991). Gene amplification and insecticide resistance. Annu. Rev. Entomol. *36*, 1–23.
10. Dumas, D.P., Caldwell, S.R., Wild, J.R., and Raushel, F.M. (1989). Purification and properties of the phosphotriesterase from Pseudomonas diminuta. J. Biol. Chem. *264*, 19659–19665.
11. McLoughlin, S.Y., Jackson, C., Liu, J.W., and Ollis, D.L. (2004). Growth of Escherichia coli coexpressing phosphotriesterase and glycerophosphodiester phosphodiesterase, using paraoxon as the sole phosphorus source. Appl. Environ. Microbiol. *70*, 404–412.
12. Copley, S.D. (2000). Evolution of a metabolic pathway for degradation of a toxic xenobiotic: the patchwork approach. Trends Biochem. Sci. *25*, 261–265.