

The 'evolvability' of promiscuous protein functions

Amir Aharoni¹, Leonid Gaidukov^{1,2}, Olga Khersonsky¹, Stephen McQ Gould^{1,2}, Cintia Roodveldt^{1,2} & Dan S Tawfik¹

How proteins with new functions (e.g., drug or antibiotic resistance or degradation of man-made chemicals) evolve in a matter of months or years is still unclear. This ability is dependent on the induction of new phenotypic traits by a small number of mutations (plasticity). But mutations often have deleterious effects on functions that are essential for survival. How are these seemingly conflicting demands met at the single-protein level? Results from directed laboratory evolution experiments indicate that the evolution of a new function is driven by mutations that have little effect on the native function but large effects on the promiscuous functions that serve as starting point. Thus, an evolving protein can initially acquire increased fitness for a new function without losing its original function. Gene duplication and the divergence of a completely new protein may then follow.

Jensen first formalized the idea that¹, under changing environments, promiscuous activity in an existing protein (also called substrate ambiguity, cross-reactivity or moonlighting activity)^{2,3} can endow the organism with a selective advantage and thereby enable its survival and further evolution. Gene duplication and mutation would then provide the increased genetic diversity that drives the evolution of a diverging new protein. Although this hypothesis is generally accepted, direct evidence for it is still scarce. To understand better the role of promiscuity in natural evolution, we used laboratory-directed evolution, a tool that not only provides access to new tailor-made protein variants but also helps to refine our understanding of protein evolution⁴.

The targets for evolution were serum paraoxonase (PON1), a bacterial phosphotriesterase (PTE) and carbonic anhydrase II (CAII). We applied a process of random mutation and selection, with the aim of increasing promiscuous activities of these enzymes. We applied only one selection pressure at a time (aiming to increase one promiscuous activity) and focused on the early evolutionary intermediates in which the first mutations that increased that promiscuous activity accumulated.

CAII is an abundant enzyme whose physiological role is to catalyze the reversible hydration of carbon dioxide. It is considered to be one of the most efficient enzymes, with almost 10^6 turnovers per second towards its native substrate (carbon dioxide, or bicarbonate). CAII has weak promiscuous esterase activity towards activated esters such as

p-nitrophenyl acetate ($k_{\text{cat}}/K_{\text{M}} \approx 10^3 \text{ M}^{-1}\text{s}^{-1}$ versus $\sim 10^8 \text{ M}^{-1}\text{s}^{-1}$ for carbon dioxide hydration)⁵. PTE hydrolyzes organophosphates, particularly paraoxon, with very high efficiency ($k_{\text{cat}} > 2,000 \text{ s}^{-1}$; $k_{\text{cat}}/K_{\text{M}} > 4 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$) and is thought to have evolved towards paraoxon, a man-made chemical that appeared on Earth only a few decades ago⁶. We identified promiscuous lactonase and esterase activities in PTE and investigated the latter. PON1 is the most studied member of a family of closely related enzymes that shares lactonase activity⁷. PON1 efficiently catalyzes the hydrolysis and the formation of five- and six-member ring lactones⁷. PON1 also catalyzes the hydrolysis of a wide range of substrates that have no apparent physiological relevance, including aryl esters and organophosphates such as paraoxon. Studies of the structure-activity relationship indicate that the active site of PON1 is tailored for lactones, and that esters and organophosphates are promiscuous substrates (O.K. & D.S.T., unpublished data).

We created genetic diversity in the genes encoding CAII, PON1 and PTE by error-prone PCR amplification under conditions that induced, on average, a few mutations per gene. We cloned the resulting gene libraries into an expression vector and used it to transform *Escherichia coli*. We plated several thousand clones from each of these libraries on agar and screened them with the target substrate. We transferred positive colonies (as determined by the appearance of a colored or fluorescent product) to 96-well plates and grew them there. We lysed the cells, assayed them with the same substrate using a spectrophotometric plate reader and compared their activity with that of the wild-type protein. We allowed PON1 to evolve towards increased activity with four different promiscuous substrates. The catalytic efficiency of wild-type PON1 with these man-made, promiscuous substrates varied from very low to medium ($k_{\text{cat}}/K_{\text{M}} = 10^2$ – $10^5 \text{ M}^{-1}\text{s}^{-1}$). Detailed experimental protocols are provided in **Supplementary Tables 1–5** online. We also allowed the very low promiscuous esterase activities of PTE and CAII with 2-naphthyl acetate ($k_{\text{cat}}/K_{\text{M}} = 480$ and $25 \text{ M}^{-1}\text{s}^{-1}$, respectively) to evolve (**Supplementary Tables 6 and 7** online).

We isolated variants of each enzyme that had higher activity with the target substrate and shuffled them (recombined them *in vitro*)⁸ to yield second-generation gene libraries, which we then screened with the same substrate. Typically, we identified a few different mutations in individual first-round variants, which were combined by the

¹Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel. ²These authors contributed equally to this work. Correspondence should be addressed to D.S.T. (tawfik@weizmann.ac.il).

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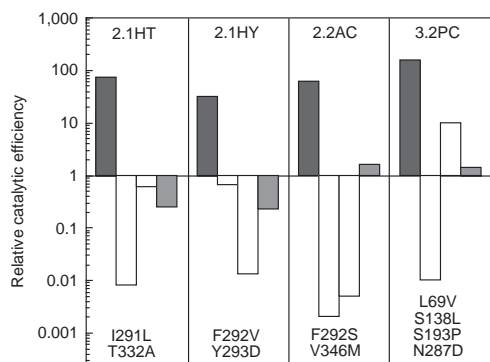


Figure 1 Changes in activities of the newly evolved PON1 variants. The ratios of k_{cat}/K_M values relative to wild-type PON1 for four evolved variants and the mutations observed in them are plotted. Black bars, the promiscuous substrate for which each variant was evolved (2.1HT was evolved for γ -butyryl thiolactone, 2.1HY for 2-naphthyl octanoate, 2.2AC for *O*-acetoxy-7-hydroxycoumarin and 3.2PC for the organophosphate 7-*O*-diethylphosphoryl-3-cyano-7-hydroxycoumarin); white bars, two other promiscuous substrates (phenyl acetate, left; paraoxon, right); gray bars, dihydrocoumarin representing the native lactonase activity of PON1. The chemical structures of these compounds are shown in **Supplementary Figure 1** online. Details regarding the selections, substrates, mutations and kinetic parameters of all mutants are presented in **Supplementary Tables 1–5** online.

in vitro recombination that followed the first and second rounds of screening (details, including the mutations and the kinetic parameters of the mutants isolated in each round of selection, are given in **Supplementary Tables 1–7** online). We continued the evolutionary process to yield further improved variants (A.A., L.G., C.R. & D.S.T., unpublished data), but in this study, we focus on the first steps after the recruitment of a promiscuous function and its improvement to provide a potential selective advantage.

For each of the six different selections described above, we isolated a range of variants whose activity was 10–150 times higher than the activity of their respective wild-type enzymes. The results of these six independent evolutionary processes are summarized in **Figures 1–3**. The selected PTE and PON1 variants also had substantial changes in activity (10–500 times higher than their respective wild-type enzyme) towards other promiscuous substrates that we did not select for. The substrate selectivities of these variants relative to those of the wild-type enzymes were as much as 3×10^4 times higher. Thus, the selection pressure for improved activity led indirectly to ‘specialization’, although no selection pressure for specialization was applied (a decrease in activity towards substrates other than the target substrate was not a selection criterion). In some cases, specialization completely reversed the selectivity towards the various promiscuous activities. For example, wild-type PON1 is an efficient aryl esterase ($k_{cat}/K_M \approx 2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ for phenyl acetate) with >100 times weaker organophosphate hydrolase activity ($k_{cat}/K_M < 10^4 \text{ M}^{-1}\text{s}^{-1}$ for organophosphates)⁹, whereas the evolved variant 3.2PC has $k_{cat}/K_M \approx 10^6 \text{ M}^{-1}\text{s}^{-1}$ for the organophosphate it was selected for and ~ 100 times lower esterase activity (**Fig. 1**). We observed similar trends with the PTE variants: the promiscuous esterase activity increased (with the selected substrate, as well as other ester substrates) and the promiscuous lactonase activity decreased (**Fig. 2**).

The most notable feature we observed in all the newly evolved variants was that the native activities changed comparatively little, in contrast to the promiscuous activities, which changed substantially.

Mutations that increased the promiscuous esterase activity of CAII by a factor of 10–40 decreased the rate of bicarbonate dehydration (the native activity of CAII) by a factor of only 1.4–2 (**Fig. 3**). In PTE, mutations that increased the promiscuous esterase activities by factors of 5–160 decreased the promiscuous lactonase activity by a factor of ~ 10 , thereby changing its selectivity by a factor of 60–1,600 relative to wild-type PTE. Native paraoxonase activity decreased only slightly (by a factor of 3.3 or less) or even increased slightly (**Fig. 2**). Likewise, the lactonase activity of the newly evolved PON1 variants barely changed (**Fig. 1**). Thus, all six evolutionary processes described above share the characteristic that mutations (few, or often a single one) that induce marked phenotypic changes in promiscuous activities have a much smaller effect (and sometimes no effect) on the native function. Conservation of the native function was not the result of a selection pressure: we applied only one selection criterion, an increase in one of the promiscuous activities of these enzymes.

Is the trend observed in our six independent experiments a general one? We explored the literature in an attempt to identify this pattern in other laboratory experiments aimed at increasing promiscuous enzymatic and binding activities of various proteins. Our findings are summarized in **Supplementary Table 8** online. They include eighteen different cases in which one to four mutations increased the promiscuous activity that was under selection by a factor of $>1,000$, on average, but hardly affected the original activity of these proteins (which decreased by a factor of ~ 3.2 on average).

The different effect of mutations on native versus promiscuous functions is particularly notable in view of the fact that these mutations occur mostly in residues that form the walls and perimeter of the active sites of PTE, CAII and PON1. The plasticity of these residues, and their potential contribution to the ability of proteins to evolve, is probably due to the fact that they are not part of the protein’s scaffold, or other core elements of the fold or of the actual catalytic machinery of the enzyme. They are on surface loops that are part of the substrate-binding pocket and have great conformational flexibility

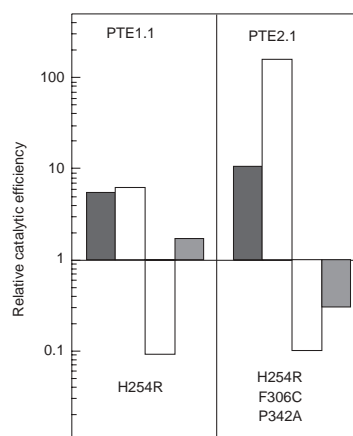


Figure 2 Changes in activities of the newly evolved PTE variants. The ratios of k_{cat}/K_M values relative to wild-type PTE for two evolved variants and the mutations observed in them are plotted. Black bars, the promiscuous ester substrate 2-naphthylacetate for which these variants were evolved; white bars, two other promiscuous substrates (carboxy fluorescein diacetate, left; dihydrocoumarin, right); gray bars, paraoxon representing the native activity of PTE. The chemical structures of these compounds are shown in **Supplementary Figure 1** online. Details regarding the selection, substrates, mutations and kinetic parameters of the mutants are presented in **Supplementary Table 6** online.

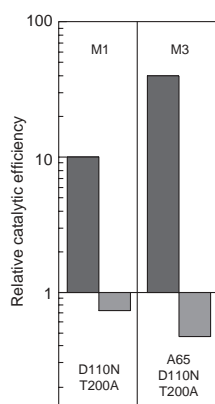


Figure 3 Changes in activities of the newly evolved CAII variants. The ratios of k_{cat}/K_M values for two evolved variants (M1 and M3) relative to wild-type human CAII and the mutations observed in them are plotted. Black bars, the promiscuous 2-naphthylacetate ester substrate (chemical structure shown in **Supplementary Fig. 1** online) for which these variants were evolved; gray bars, the native activity of bicarbonate dehydration. The detailed kinetic parameters and mutations are listed in **Supplementary Table 7** online.

(**Fig. 4**). A notable example of this principle is α -lytic protease, in which a single amino acid substitution increased the activity towards promiscuous substrates by a factor of 10^5 but reduced the native activity by a factor only 2 (ref. 10); the structural flexibility of the substrate-binding loops allowed this large shift in the selectivity of this enzyme and of its family members^{10,11}. There may also be fundamental differences between the mode of binding of the native substrate, which is typically mediated by several independent, enthalpy-driven interactions, versus that of the promiscuous substrates, in which hydrophobic and other entropy-driven interactions are important¹².

Do promiscuous activities have a key role in protein evolution? Numerous promiscuous activities have been identified^{13,14}, including ones that are markedly dissimilar to the native function¹⁵. A protein may use one conformation to carry out its native function and an alternative conformation to carry out a promiscuous function^{3,16}. Although promiscuous activities are often orders of magnitude lower than the native activity, they may provide a selective advantage^{15,17,18}. Our results suggest that promiscuous activities have another distinct inherent advantage: they have an unusual plasticity, or lack of robustness, that is not seen with the native function. Robustness of the native function can be acquired in the course of the evolutionary

process¹⁹, but the promiscuous functions are latent and were never under selection pressure. Yet robustness may well be a consequence of the high proficiency of the native activity and of the structural features described above, rather than a selectable trait in itself.

The observation that substantial changes in the promiscuous functions of a protein need not come at the expense of its native function may also explain why enzymes can rapidly acquire mutations that lead to loss of inhibition by drugs (drug resistance) but barely affect their enzymatic function. Drug binding is promiscuous by definition: the enzyme never evolved, or adapted, for drug binding. But the native function can tolerate the same mutations that diminish the promiscuous drug binding, as shown here. The characteristics of drug resistance^{20,21} seem to be similar to the ones observed here: mutations that confer drug resistance are in substrate-binding loops, rather than in the catalytic residues or the protein's scaffold (**Fig. 4**), and adaptability towards drug binding involves conformational flexibility^{10,11}.

Multi-specific enzymes, such as PON1 with its myriad promiscuous activities and broadly defined native substrate, may resemble ancestral proteins¹ or evolutionary nodes²² and intermediates²³. The fact that such enzymes can rapidly diverge and yield highly proficient and selective variants (as shown here for four different activities of PON1) may further support their key role in evolution. Moreover, wild-type PTE and CAII are 'specialized' towards one activity. Yet their evolved variants are multi-specific, or 'generalized', because they have gained new activities but still maintain their native ones. This observation is in agreement with the hypothesis that protein evolution towards a new function involves the transitions from a specialized enzyme into a generalized intermediate and, ultimately, a new, 'respecialized' enzyme²³. The divergence of new proteins could follow this route: initially, a gene acquires a beneficial mutation that renders it generalized by increasing the protein's promiscuous activity to a level sufficient for survival while maintaining the original activity largely intact. Gene duplication, and the divergence of a completely new gene (with respect to sequence and function), then follow²⁴.

A more speculative aspect of this work concerns the concept of evolutionary adaptability or 'evolvability', the capacity of biological systems to evolve. This concept raises much controversy, especially in its wider context²⁵, particularly regarding whether it is a selectable trait²⁶. But it is beyond dispute that evolutionary processes depend on the benefit of rare favorable mutations, although these benefits can be quickly erased by deleterious mutations that are far more frequent than beneficial ones²⁷. Thus, evolution depends on two critical, and seemingly conflicting, features: (i) a reduced lethality of mutations (robustness) and (ii) the induction of new phenotypic traits by a

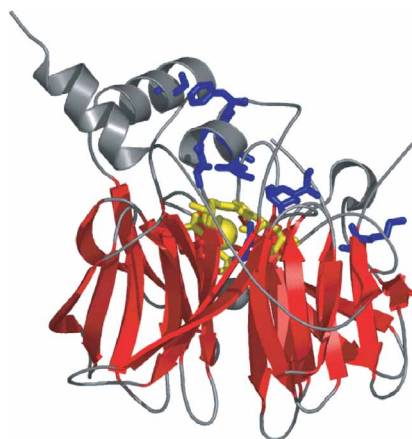


Figure 4 The location of the selectivity-changing mutations observed in the directly evolved PON1 variants. The six-bladed β -propeller scaffold is shown in red, with the structural calcium in its central tunnel shown in gray; the catalytic machinery (the top calcium atom and its ligating residues, and His115) are shown in yellow. The residues that give rise to marked changes in the promiscuous activities of directly evolved PON1 but barely change its native lactonase activity (**Fig. 1**) are shown in blue. They are not part of the scaffold of PON1 or of the catalytic machinery that is linked to this scaffold. Rather, the selectivity-changing residues are on surface loops, and helices connected to these loops, that 'decorate' the scaffold and form the substrate-binding pocket. These loops tend to have great conformational flexibility. Some of the mutations observed in PON1 are in a loop that is disordered and not visible in the crystal structure (residues 72–79)³⁰. The PON1 structure (PDB entry 1V04)³⁰ was drawn with PyMol.

relatively low number of mutations (plasticity)²⁵. Previous work has identified several mechanisms by which nature hedges between the cost and benefit of mutation. For example, the rates and patterns of genetic and phenotypic variability may vary with the severity of the selection pressure^{27–29}. Our hypothesis provides a possible solution to these conflicting features at the single-protein level: rapid adaptability is inherent to the promiscuous, accidental functions of the protein, and their plasticity need not be at the expense of the protein's original activity.

METHODS

We used genes encoding the following proteins as starting points for directed evolution: human CAII cloned into pET20b (Novagen), a PTE variant S5 cloned into pMAL-c2x (C.R. & D.S.T., unpublished data) and a recombinant PON1 variant (G3C9) cloned into a modified pET32b vector⁹. We prepared libraries by error-prone PCR amplifications of these genes, at biased dNTP concentrations in the presence of manganese chloride, and by wobble-base PCR as previously described^{9,30}. We transformed *E. coli* cells grown on agar plates with the resulting CAII, PTE and PON1 libraries. We replicated the plates and screened them for 2-naphthylacetate hydrolysis using Fast Red to detect the 2-naphthol product as previously described⁹. We screened PON1 libraries with three additional substrates: 2-naphthylacetate was screened by Fast Red detection, and hydrolysis of the organophosphate substrate 7-*O*-diethylphosphoryl-3-cyano-7-hydroxycoumarin and the ester substrate *O*-acetoxy-7-hydroxycoumarin were screened by detecting the fluorescent coumarin products under a 360-nm ultraviolet lamp³⁰. Positive clones identified from each screen were grown in liquid medium in individual wells of 96-well plates, lysed by BugBuster (Novagen) and assayed for a variety of different substrates using a spectrophotometric plate reader^{9,30}. We extracted plasmid DNA from all clones that had a significantly higher rate of hydrolysis relative to their respective starting gene (typically 3–10 times higher) and subjected it to DNA shuffling⁸ to generate the second-generation libraries. Positive variants isolated from the second round of screening were either reshuffled (PON1) or randomly mutagenized (CAII) to yield the third-generation libraries that were screened with the same substrate.

We overexpressed the best PON1 and CAII variants from the last round of screening in Origami B (Novagen) *E. coli* cells and purified them by affinity chromatography on Ni-NTA agarose (PON1 variants)⁹ or agarose-coupled *p*-aminomethyl benzene sulphonamide (CAII variants). We expressed the PTE variants in DH5 α cells and purified them on an amylose resin. We assayed the purified newly evolved variants at a range of enzyme (0.005–4 μ M) and substrate concentrations (typically 0.3–3 \times K_M). We measured the rates of product formation spectrophotometrically in 96-well plates using 200- μ l reaction volumes as described^{9,30}. When necessary, we corrected rates for the rate of spontaneous hydrolysis of the substrate in the absence of enzyme. We derived kinetic parameters by fitting the initial rates of product release to the Michaelis-Menten model. Rates of hydrolysis for substrates with limited aqueous solubility had to be measured below the K_M concentrations, and k_{cat}/K_M values were derived directly ($k_{cat}/K_M = V_o/[E]_0[S]_0$). The CAII variants were also assayed for bicarbonate dehydration (Supplementary Table 7 online). Other details regarding each of the six directed evolution experiments described above are given in Supplementary Tables 1–7 online.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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