Glutathione Transferases and Serine Proteases: From Probing Mechanism of Enzyme Catalysis by Rational Protein Engineering to Evolutionary Design of Enzyme Function

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Summary

Research of the past decade has demonstrated that the use of enzymes and whole-cell biocatalysts is environmentally friendly, economical and surprisingly, faces few barriers when applied in organic syntheses. In nature, enzymes evolved to function within a living system and may not exhibit features desirable for large-scale *in vitro* syntheses. Thus, protein engineering has the potential to dramatically enhance enzyme performance in a wide variety of unusual – but technologically important – environments. Site-directed, cassette-mutagenesis and construction of chimeric enzymes commonly used in rational protein engineering are important strategies which reveal the structure-function relationship of a given enzyme. Notably, construction of hybrid enzymes has become increasingly important in the rational design of novel biocatalysts with desired properties and activities. However, "irrational" protein design based on random mutagenesis technologies or the combination with directed evolution approaches has truly revolutionized the generation of functional biological molecules.

Key words:
rational design, directed evolution, screening, enzyme mutants, new catalytic functions.

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1. Introduction

Enzyme engineering is commonly perceived as a process leading to the generation of mutant proteins with predetermined enzymatic characteristics, such as improved stability, modified pH optima or improved catalytic efficiency. Until the 1970s, the only way to address the importance of "essential" amino acid residues and the individual side chains in the enzyme's active site was to modify them using chemical reagents. Nowadays, mutagenesis is not performed on the enzyme itself, but on the gene (DNA segment) that encodes a particular enzyme, and thus, takes advantage of a plethora of available DNA manipulation methodologies.

The rational approach challenges our understanding of protein biochemistry as it relies on a detailed knowledge of an enzyme's structure, function and catalytic mechanism (1, and references herein). A process of rational enzyme redesign can target single amino acids (e.g., by site-directed or cassette-mutagenesis) or can lead to a generation of hybrid (chimeric) enzymes from two or more parents. Notably, sometimes the removal of "essential" amino acids from the enzyme active site may not abolish its activity, but may instead alter its catalytic mechanism significantly (2). *In vitro* generation of enzyme chimeras has been shown to be useful in the alteration of both non-enzymatic and enzymatic properties, as well as in understanding the structure-function relationship (3). Moreover, fragments of a chimeric enzyme can serve as building blocks of novel proteins, which could acquire the ability to catalyze reactions not observed in nature. Still, with the exception of a few remarkable successes (4-6), the creation of useful biocatalysts solely by rational design has remained a true challenge. The effects of multiple mutations on protein function are not easily predictable even with the available structural data. Additionally, structural prediction and a site-directed mutagenesis of target residues tend to be resource-intensive and time-consuming. These obstacles led to the development of new technologies that have made a remarkable impact on the field of protein engineering.

Although strategies for generation of new enzyme functions by *in vivo* directed evolution have been recognized for a long time (7), it is only recently that the statistical element of natural evolution has been utilized to explore functional sequence space *in vitro*. Initially, *in vitro* directed evolution of proteins relied exclusively on random mutagenesis alone (e.g., error-prone PCR). Although important, this random mutagenesis approach still did not satisfy the statistical complexity of natural evolution as it lacked nature's combinatoriality. Recently developed directed evolution strategies involving reiterative random mutagenesis *and* recombination overcame the problems of simple mutagenic-PCR, and thus, became increasingly important for generation of enzymes with desired activities and properties (8,9).

As an example of rational enzyme design and its significance for understanding of catalytic mechanism of a given enzyme, in the first part of this review, I will briefly discuss my studies on glutathione transferases (GSTs, EC 2.5.1.18). The second part of the review adresses the development of a molecular evolution strategy

for creation of enzymes with improved or new specificities, and its application to the generation of broad esterolytic serine proteases.

2. Rational Enzyme Design

GSTs are multifunctional enzymes that catalyze the conjugation of glutathione with a wide range of electrophilic compounds (10,11). It is thought that the GST superfamily evolved from a common ancestral gene *via* gene duplication, exon shuffling, and gene conversion events. Individual GST isoforms display distinct, but overlapping substrate specificities. Since many highly reactive chemicals detoxified by GST metabolic pathway result from normal cellular oxidative metabolism, expression of GSTs provides cells and organs with an efficient scavenging mechanism (10,11).

A classical protein engineering approach based on site- and oligonucleotide-directed mutagenesis was used for determination of amino acid residues that are involved in the catalytic mechanism of GSTs. Additionally, structural bases for the high catalytic activity of several GSTs with 4-hydroxyalkenals were probed by generation of hybrid enzymes.

2.1. Dissecting active-site residues for glutathione transferases

Prior to the publication of the structure of the pig lung class Pi GST (12), no definitive information was available on active-site residues for any GST. Although earlier studies suggested that three arginine-residues (Arg-13, -20, and -69) are important for catalytic activity of Alpha GST2 (13), no clear evidence for the involvement of other amino acid residues in the catalytic mechanism was available. Our amino acid sequence analysis revealed conservation of a tyrosine residue 7 (Tyr-7) not only in the class Pi of GSTs, but also in several other GST classes (for nomenclature, see 10). Importantly, the analysis of the structure data of the pig lung class Pi GST explicitly pointed at the hydroxyl residue of Tyr-7 as potentially involved in the formation of a hydrogen bond with the sulphonyl group of the glutathione sulphonate bound to the G-site of the enzyme. Thus, we proposed that the hydroxyl group of Tyr-7 might interact with the thiol group of gluthatione and thereby facilitate its nucleophilic attack on the second, electrophilic substrate molecule.

In order to test this hypothesis, the equivalent tyrosine residue (Tyr-8) in the homologous human class Pi enzyme (GST P1-1) was converted into phenylalanine by means of oligonucleotide-directed mutagenesis. The only difference between the wild-type (WT) GST P1-1 and the generated mutant enzyme was the lack of the hydroxyl group on the phenolic moiety of the amino acid replacing Tyr-8. The kinetic study of the WT GST P1-1 and the corresponding Tyr-8-Phe mutant demonstrated the primary role of Tyr-8 (WT GST P1-1) to be in catalysis, not in the binding

of the substrates. Notably, the participation of the hydroxyl group of Tyr-8 in the catalytic mechanism of human class Pi GST P1-1 had not been previously shown. Our study revealed that Tyr-8 has to be protonated in order to be active in catalysis. Thus, the possibility of participation of ionized Tyr-8 in ionization of gluthatione, which we formerly considered, was ruled out (14).

The described example of the human GST P1-1 mutagenesis contributes to the commonly recognized significance of rational approach in elucidation of the catalytic role of active-site amino acids. Over the years, many books and reviews (2,15, and references herein) have been written on the topic to which I refer the readers.

2.2. Hybrid glutathione transferases

Another part of the study on structure-function relationships of the GSTs superfamily was based on the strategy of generating enzyme chimeras. In order to probe the structural basis for the difference in substrate specificities of rat GST subunits 1 and 8 (the Alpha class GST), catalytically functional chimeras were created by the replacement of the 5'-region of subunit 8 cDNA by the corresponding cDNA sequence of subunit 1. After the expression of the generated hybrid enzymes in *Escherichia coli*, all (four) of them were catalytically active. However, the high specific activity of subunit 8 with 4-hydroxyalkenals was lost when the N-terminal portion of the subunit was replaced by the corresponding segment of subunit 1 (16). In an attempt to restore the high 4-hydroxyalkenal activity of this chimera, point mutations converting individual amino acids to the residues found in the wild-type subunit 8 were made. Residues in the vicinity of Tyr-9 were selected, but neither the His-8 to Tyr nor the Asn-11 to Gln mutations restored any of the high 4-hydroxyalkenal activity of subunit 8 (unpublished data). Although all the possible mutations were not tested, it is highly probable that the characteristic catalytic properties of subunit 8 may *not* reside specifically in the N-terminus of the protein, but result from more subtle interactions between domains or subunits of the enzyme. This issue brings us to the dilemma of traditional protein engineering based on site- and oligonucleotide-directed mutagenesis which quite often underscores the sophistication of enzymes' catalysis and their overall functional plasticity. Some solutions to the previously mentioned dilemma were brought by recent advancements in "irrational" strategies of protein engineering.

3. "Irrational" Enzyme Design

This section highlights the rationale and the key aspects of my work on "irrational" design of enzymes. My early studies on evolution of GSTs were primarily focused on the development of experimental procedures for random DNA mutagene-

sis by error-prone PCR, as well as on recombination of GSTs genes using PCR (unpublished data) and are not discussed here. My work on directed molecular evolution of hydrolases (unpublished data) that includes the recent study of the serine protease, subtilisin E, is not only academically important, but should also lead to practical applications of the evolved mutant enzymes, for example, to their use in industrial sugar polymer syntheses.

Sugars are a particularly interesting class of polyfunctional compounds. They are biologically relevant and contain a large number of hydroxyl groups that are nearly indistinguishable chemically. Their incorporation into polymers is a natural event leading to a tremendously diverse universe of polysaccharides. Sugars have also been modified enzymatically and incorporated into polymers quite distinct from polysaccharides. Polysaccharides form the basis for many commercially relevant materials, including drugs, biopolymers, and sweeteners.

By using the principle of micro-reversibility, lipases and proteases can be applied as biocatalysts in sugar polymer syntheses in nonaqueous media (17). One such enzyme is the serine protease, subtilisin E. Subtilisin E is well suited for directed molecular evolution, because in addition to its natural amidase activity, the enzyme possesses a relatively weak esterolytic activity on sugar ester substrates. Many properties of subtilisin have been altered by various protein-engineering approaches; however, none of those studies addressed the *esterolytic* activity of the enzyme. I am particularly interested in determining the extent to which one can change the amidase and esterase functions by manipulating subtilisin at the molecular level. Towards this, I endeavored to transform this protease into a broadly selective esterase. In the process, a strategy for molecular evolution of subtilisin, which is comprised of several unique steps, was also developed. Briefly, the strategy is based on the periplasmic expression of subtilisin libraries in *Escherichia coli* (18). The key component of the strategy involves cell-based kinetic screening assays for esters' hydrolysis. An efficient and fast combinatorial process for mutagenesis of subtilisin E genes was developed for generation of mutant libraries. I refer readers to the literature for specific details, as a part of this study has been published (18,19).

3.1. Generation of Subtilisin E Mutant Libraries

A protocol for generation of low-level point-mutation libraries that significantly improved the overall quality of the created libraries was developed through this study such that a relatively small number of screened clones was suitable to identify desired enzymatic traits (18). The key difference between the developed protocol and the ones described in the literature is that the second mutagenic PCR and shuffling was performed using the mixture of already once mutated and shuffled subtilisin genes as the DNA template. Thus, one intermediate step of transformation

and screening for amidase and esterase activities was omitted. Generation of libraries containing low rates of random nucleotide substitutions is dictated by both experimental limitations and theoretical considerations. When a low rate of mutations is used, a large fraction of all the possible amino-acid substitutions ("sequence space") may be represented in a relatively small library. Also, a low-rate mutagenesis is considered necessary to maintain the fraction of deleterious mutations at a tolerable level (20,21). The iterative screening of relatively small libraries of mutants with a low frequency of nucleotide substitutions has proven to be very effective for the functional improvement of numerous proteins (8,22).

3.2. Screening of Enzyme Libraries

Several different *cell-based* qualitative and quantitative assays that facilitate screening for esterolytic activity of subtilisin were developed through the study under discussion. The most significant ones are the cell-based *kinetic* assays that permit measurement of enzymes' activity using microtiter plates (18), because they can be further developed as high-throughput screens. Especially valuable is the assay developed for the screening of enzymatic sugar-esters hydrolysis (18). This assay is based on the generation of protons during the respective ester hydrolysis that are detected by a relevant pH indicator. Moreover, this universal assay can be applied for screening of any catalytic activity coupled to a change of pH. The introduction of the amidase to esterase activity *ratio of initial reaction rates* was a novel and critical parameter assuring accuracy of all the developed cell-based kinetic screenings. Calculating ratios eliminated the influence of promoter-dependent and gene-dependent differential expression levels on enzyme activity for the different mutant enzymes generated (18,19).

3.3. Broad Esterolytic Subtilisins

Using the developed strategy (18,19), three mutant subtilisins with increased ratios of esterase to amidase activities on two structurally distinct esters were evolved. The best mutant showed a 70-fold and 9.6-fold swing in the ratio of esterase/amidase activity for Phe-*p*-nitrophenyl ester and sucrose 1'-adipate, respectively, when compared with WT subtilisin E. As the two ester-substrates are structurally distinct, a more general esterolytic subtilisin was evolved and this may have important synthetic applications. Evolution of a broad esterolytic activity for a proteolytic enzyme has not been previously reported. Notably, the increased esterase activity was not obtained at the expense of overall amidase activity (18).

Subsequently, detailed analysis of the three evolved mutants at the primary, secondary and tertiary structure levels was performed. A total of 16 amino acid resi-

dues were changed by the developed molecular evolution strategy when compared to the three parents globally. A new mutation (Val-143-His) common to all three mutants that appears to be pivotal in converting subtilisin E into a general esterase was identified. A simple structure analysis of all three mutants using the RASMOL program led to an intriguing observation that the position of the new mutation lines the binding pocket for the peptide substrate. Thus, it is possible that the presence of the new amino acid in the binding pocket of the three mutants could facilitate improved binding (or positioning) of the respective *ester* substrates (18). More advanced molecular modeling of one of the mutants is currently being conducted.

The parental DNA makeup of the mutants was also determined. In contrast to classical breeding, more than two parents can contribute to each of the progeny depending on the molecular breeding format used. Interestingly, all three parents contributed to the final genetic makeup of two mutants, and only two parents contributed their genetic material to the third mutant (18).

4. Future Prospects

Long standing interest in enzymes and their function at the molecular level will continue to motivate research in diverse science and mathematics fields. Enzymes are fascinating catalysts capable of accepting a wide array of complex molecules as substrates. They are exquisitely selective in catalysis on unparalleled chiral (enantio-) and positional (regio-) substrates. As a result, enzymes can be used in both simple and complex transformations without the need for the tedious blocking and deblocking steps that are common in enantio- and regioselective organic syntheses. This high selectivity of enzymes also makes reactions efficient and leads to a fewer byproducts, the key feature of an environmentally friendly alternative to conventional chemical catalysis. It seems apparent that future biocatalytic processes will not be limited to the currently available technologies or by the availability of natural biocatalysts. Proteins will be more commonly used as a scaffold to create new proteins and enzymes. Such new enzymes, for example, will have potential to become components of designed metabolic pathways, which subsequently would lead to synthesis of novel biomaterials or drugs. Thus, for the benefit of the desired progress, a combination of both "rational" and "irrational" approach should be the driving force in generation of new enzymes.

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