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Review

Behind the Scenes of PluriZyme Designs

Ana Robles-Martín [1,2], Sergi Roda [1,3], Rubén Muñoz-Tafalla [1,2] and Victor Guallar [1,4,*]

[1] Barcelona Supercomputing Center (BSC), 08034 Barcelona, Spain; sergi.roda@nostrumbiodiscovery.com (S.R.); ruben.munoz@bsc.es (R.M.-T.)
[2] Department of Biochemistry and Physiology, School of Pharmacy and Food Sciences, Universitat de Barcelona (UB), 08028 Barcelona, Spain
[3] Nostrum Biodiscovery S.L., 08029 Barcelona, Spain
[4] Institució Catalana de Recerca i Estudis Avançats (ICREA), 08010 Barcelona, Spain
* Correspondence: victor.guallar@bsc.es

Abstract: Protein engineering is the design and modification of protein structures to optimize their functions or create novel functionalities for applications in biotechnology, medicine or industry. It represents an essential scientific solution for many of the environmental and societal challenges ahead of us, such as polymer degradation. Unlike traditional chemical methods, enzyme-mediated degradation is selective and environmentally friendly and requires milder conditions. Computational methods will play a critical role in developing such solutions by enabling more efficient bioprospecting of natural polymer-degrading enzymes. They provide structural information, generate mechanistic studies, and formulate new hypotheses, facilitating the modeling and modification of these biocatalysts through enzyme engineering. The recent development of pluriZymes constitutes an example, providing a rational mechanism to integrate different biochemical processes into one single enzyme. In this review, we summarize our recent efforts in this line and introduce our early work towards polymer degradation using a pluriZyme-like technology, including our latest development in PET nanoparticle degradation. Moreover, we provide a comprehensive recipe for developing one's own pluriZyme so that different laboratories can experiment with them and establish new limits. With modest computational resources and with help from this review, your first pluriZyme is one step closer.

Keywords: pluriZyme; rational design; active site; protein engineering; computational chemistry

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1. Introduction

Enzyme engineering is the design and construction of new enzymes or the modification of existing ones with desired properties for specific practical uses. It has widely proven its importance in a vast number of applications, with a significant recent increase in the number of studies in both academia and industry [1–3]. Such an increase is largely based on the advantages of using proteins to catalyze reactions, mainly in the form of sustainable and greener alternatives to chemical catalysts. In addition, the constantly growing supply of new enzymes derived from bioprospecting campaigns, along with their adaptable nature, makes them a very interesting raw material. In this sense, enzyme engineering aims to improve the performance of these natural catalysts in terms of properties such as activity, specificity, and stability.

We find a wide diversity of methodologies developed for the improvement of enzymes [1]. On the experimental side, a plethora of rational mutagenesis [4–7] and directed evolution [8–10] techniques have been at the center of attention. We are interested, however, in computational techniques for enzyme engineering, among which we find several recent approaches, such as de novo design of active sites in protein scaffolds by computational rational design that requires knowledge of the general empirical rules [7,11,12], the use of artificial intelligence to find the best mutations to improve stability or a specific form of activity [13,14], and atomistic protein dynamics studies using MD techniques [15–17].

In our laboratory at the Barcelona Supercomputing Center, we have recently introduced a different approach based on adding one or multiple active site(s) to an existing enzyme, a concept named pluriZyme [4,18,19]. Such an approach has the additional difficulty of keeping the native activity as intact as possible (see, for example, a good recent review on this topic [20]). In this way, the addition of an active site with the same activity as the wild-type one may improve the catalytic performance of the enzyme by increasing the activity and/or substrate scope [19–21]. On the other hand, adding an active site with different activity allows a single enzyme scaffold to perform a cascade reaction [21,22]. The pluriZyme concept is in line with recent efforts in designing proteins bearing multiple enzyme centers using different approaches: (i) the self-assembly of histidine–tyrosine peptides mimicking a catalytic microenvironment [23]; (ii) the use of a non-catalytic protein scaffold (a lactococcal multidrug resistance regulator) resulting in two abiological catalytic sites [24,25]; (iii) the coupling of metal nanoparticles to gain catalytic properties [26–29]; and (iv) cofactor modification for a new host that incorporates both a mimic of NADH and a flavin analog [30] (see Figure 1 for an overview of some strategies for engineering multiple catalytic centers).

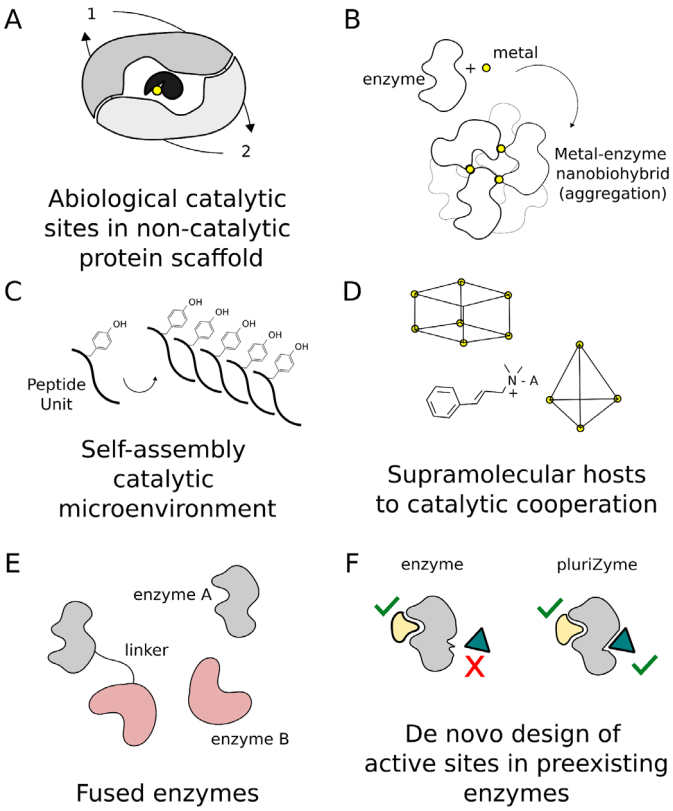

Figure 1. Strategies for engineering multiple catalytic centers. This figure provides an overview of strategies employed to engineer multiple catalytic centers, showcasing the versatility of these diverse methodologies. (**A**) Incorporation of abiological catalytic sites within a non-catalytic protein scaffold (1 refers to the initial substrate and 2 to the product of the first reaction). (**B**) Formation of metal–enzyme aggregations between metallic elements and enzymes to create multifunctional catalytic centers. (**C**) Construction of catalytic microenvironments through peptide self-assembly. (**D**) Use of supramolecular hosts to facilitate catalytic cooperation. (**E**) Fused enzymes. (**F**) PluriZyme strategy ($\surd$ means that the binding site can catalyze the substrate, while $\times$ means that the binding site can not catalyze the substrate).

The design of pluriZymes has been based on adding catalytic triads capable of introducing esterase hydrolytic biochemistry [4,18]. The engineering effort resides on mutating amino acids (up to three, although we do aim to reuse existing residues) to form a hydrogen-

bonded catalytic triad, typically introducing serine, histidine, and aspartic or glutamic acid. Additional mutations might also be necessary to better accommodate the triad, to open the pocket, or to introduce an oxyanion hole. Importantly, using covalent suicide inhibitors, we have been able to introduce additional forms of activity other than hydrolysis, such as oxidation activity [21]. Inserting an extra active site allows the inclusion of different chemistries in a single protein scaffold, reducing the costs and optimization of protein expression. Thus, pluriZymes could become part of the next generation of biocatalysts for a more sustainable future, as recognized through their portrayal on the cover of *Nature Catalysis* [21]. They can have a wide range of applications, including in industrial processes; the production of fine chemicals, food, or pharmaceuticals; and the degradation of pollutants. As such, some of our more recent designs show high activity in the degradation of polymers, including polyethylene terephthalate (PET) nanoparticles and xylane. For instance, their enhanced enzymatic activity towards PET degradation could be applied in the development of environmentally friendly methods for recycling PET-based plastics, contributing to a reduction in plastic waste. Similarly, their proficiency in xylane degradation holds promise for applications in the bioconversion of lignocellulosic biomass, addressing challenges in sustainable biofuel production and biorefinery processes.

In this paper, we summarize all pluriZymes developed to date, emphasizing the best practices and what occurs behind the scenes during the engineering process. We also underline two of the more recent developments, designed to improve polymer degradation. Overall, we aim to provide the pluriZyme-making recipe so that any laboratory with a bit of experience in computational modeling can reproduce its own designs.

2. How to Develop a PluriZyme

PluriZymes are proteins with more than one active site capable of enzymatic catalysis, where at least one of these has been designed by protein engineering. In this procedure, rational design plays a crucial role. The overall workflow for creating a de novo pluriZyme follows the steps summarized in Figure 2.

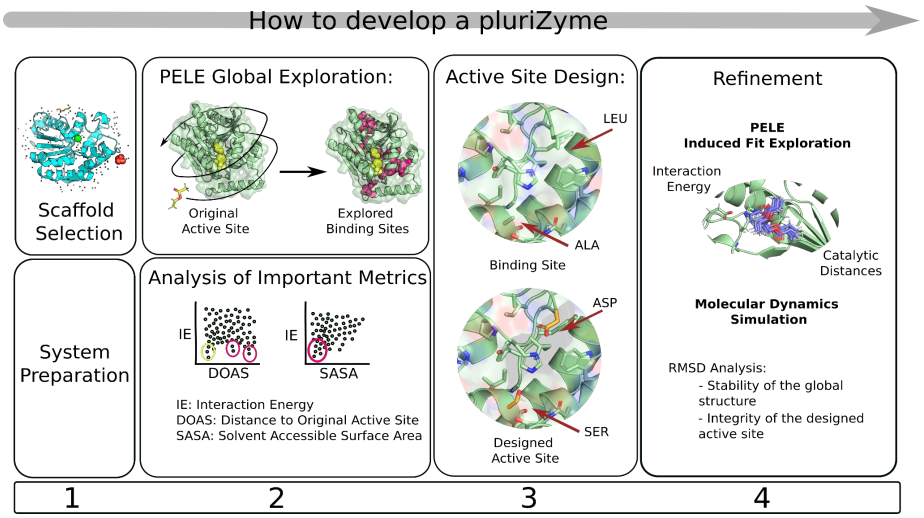

Figure 2. Workflow for the development of pluriZymes.

2.1. System Preparation

The first step always consists of knowing the system to prepare it adequately: the cellular location (membrane or soluble protein), number of system subunits, stability at different pHs and temperatures, cofactors, and modified or essential residues. The next step starts from a 3D protein structure that ideally comes from a resolved crystal or nuclear magnetic resonance (NMR). We can also start from a model thanks to homology modeling and, more recently, to the breakthrough of deep learning structural builders, such as AlphaFold 2.0 [31]. Moreover, a model structure can add missing or omitted parts to the

experimental structure. Finally, the hydrogen bond network should be optimized; we typically use Maestro's Protein Preparation Wizard [32] to correct the protonation states depending on the pH of interest.

2.2. Binding Site Search

The second step, since we want to design new active sites in a protein scaffold, involves selecting a substrate(s) to simulate the active-site binding event. Once the substrate is selected, we prepare it with a quantum mechanics calculation in an implicit solvent and obtain the ESP charges with Jaguar [33]. Now that both the protein and the substrate are prepared, we perform PELE global exploration [34] (also known as SiteFinder), aimed at identifying potential binding sites (other than the wild-type active site if we are working with an enzyme) preexisting in the natural protein scaffold. PELE (Protein Energy Landscape Exploration) is our in-house Monte Carlo software capable of mapping complex intermolecular biophysical problems, such as global ligand migration, local induced fit, etc. [35]. As PELE uses implicit solvent (OBC or SGB), it tends to close the protein because the system maximizes stabilizing contacts between residues. Therefore, adding a series of constraints to the initial structure is necessary, generally with a force of 5 kcal/(mol·Å^2) to one residue every 5 or 10 residues along the sequence. The metrics that most interest us at this point are the solvent-accessible surface area (SASA) of the substrate and the binding energy throughout the simulation. If we observe a local energy minimum, it might indicate that the substrate, during its exploration, has found a binding site—a cavity in which it can remain for a long time due to stabilizing intermolecular interactions. If we do not find a potential new binding site, we must consider whether we want to continue working with the system by designing a binding site—by opening/enlarging some nascent cavity, for example. For this, we might still want to identify some transient site by selecting the best interaction energies (nascent binding site) and enlarge it, similarly to what we performed recently to increase promiscuity in an esterase [36]. While we use PELE in our laboratory, other software capable of describing potential binding sites in an enzyme could be used at this point, such as a combination of docking and molecular dynamics [37,38] and even AI-based tools [39].

2.3. From Binding Site to Active Site

The third step involves the conversion of the binding site into an active site, involving amino acid mutagenesis to introduce a catalytic triad. When carrying out mutagenesis, we consider several factors, including: (i) the conservation of the residues that we want to mutate in other homologous sequences, (ii) prioritizing mutations to residues of the same category, (iii) prioritizing residues close in sequence space (not only in Euclidean space), and (iv) amino acid recycling. Regarding this last point, we prioritize catalytic triads where we use a wild-type acidic residue, since adding negative charges to a protein might have a detrimental effect. The presence of negative charges on acidic residues (such as aspartate or glutamate) can lead to unfavorable interactions with the carbonyl oxygens of the protein backbone. The extent of destabilization may vary depending on the distance to a neighboring backbone carbonyl. The introduction of a negative charge has the potential to impact stability, exerting both local and global effects [40].

Our goal is to obtain an active site with well-organized catalytic triads (with proper distances and angles) and with the least possible number of mutations; for this, as many combinations as possible of potential catalytic triads are made.

Next, we perform a refinement simulation of the newly designed active site(s) using the likelihood of catalytic encounters using PELE simulations. We explore the movements of the substrate inside the cavity mapping and how mutations affect its binding energy profile and localization. Ideally, we want the substrate to remain in the binding site with similar or even better substrate-binding energies (the intermolecular interaction energy provided by PELE). Eventually, we analyze the distances between the reactive atoms of the substrate and the catalytic residues, accounting for all the catalytic events that can be

observed. We consider a catalytic event to occur when the catalytic distances between the substrate and the reactive residues are below ~4 Å. In an ester hydrolase, for example, it would correspond to the distance between the ester carbon and the alcoholic oxygen from the catalytic serine. Moreover, the rest of the catalytic residues must adopt reasonable distances and angles (including those that participate indirectly, such as the oxyanion holes in the case of hydrolases). The number of catalytic poses helps us to rank different catalytic constructs and assess the activity of the newly designed active site.

2.4. Refinement

Designs with better stability, binding energy, and number of catalytic events are further refined with molecular dynamics simulations to give more robustness to the engineering process. The goal is to double check the stability of the global structure, the integrity of the newly designed catalytic triad, and that the substrate does not abandon (escape) the active site. After the MD analysis, which is based on distance measurements and RMSD, a ranking of the mutants is established, and the best ones are selected for experimental validation.

2.5. Automation of PluriZyme Design

Out of the different steps involved in the pluriZyme design recipe (shown in Figure 2), the third step, active-site design, is the one that has involved the most human intervention and intuition in the different pluriZymes designed to date (see below). In order to alleviate the non-deterministic nature of the procedure, we developed automated software.

Toward this goal, a new Monte Carlo software program, AsiteDesign, was implemented and published recently [41]; it is mainly based on the pyRosetta library [42]. The algorithm starts by selecting a set of positions defined by the user, which should identify those residues around the identified alternative binding site. Then, all these positions are allowed to be mutated to create randomly different combinations of catalytic triads along the simulation, and the variants are ranked based on an energy metric after every iteration (Figure 3). Distance constraints are enforced during the simulation to provide the correct distances to the introduced catalytic residues. Ligand sampling is included in the simulation, as we believe it helps find the optimal solutions and ensures that the active site will have activity against that molecule. To enhance the sampling, adaptive reinforcement [35] and simulated annealing protocols are implemented. The number of designs increases by n!/(n − k)! (where k = 3 in catalytic triads). Thus, when the number of residues is 10 (n = 10), the number of possible catalytic triads is 720. This combinatorial problem is what AsiteDesign tries to address by intelligently sampling the possible combinations and outputting the best ones ranked by an energy metric.

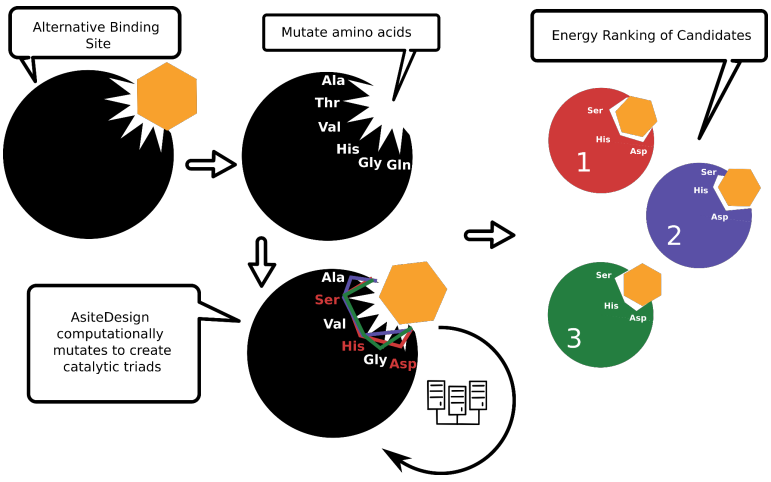

Figure 3. Simplified scheme of AsiteDesign workflow. The colors of the enzyme scaffold represent different mutants that AsiteDesign returns as possible options to insert a catalytic triad. The digits refer to the energy ranking of these designs.

AsiteDesign gives reliable catalytic triads without requiring the user to design them. In fact, the used benchmark was an esterase, and the catalytic residues of the system were mutated to Ala to see if the protocol could recover the original active site. AsiteDesign recovered the wild-type active site as the top-ranked solution. Likewise, it gave alternative catalytic triads of which the second-best option was assayed experimentally and had hydrolase activity [41]. We have made the code freely available to everyone: a container version is available at https://github.com/BSC-CNS-EAPM/AsiteDesign-container, accessed on 21 December 2023; its usage, however, requires a Rosetta license (which is free for non-commercial use).

3. Successful Designs of PluriZymes

Currently, and in close collaboration with the laboratory of Manuel Ferrer (CSIC-ICP in Madrid), we have already developed six pluriZymes, and we are working on six additional ones, mostly under the FuturEnzyme EU project (https://www.futurenzyme.eu, accessed on 21 December 2023), see Table 1. The first one, developed in 2018, included the addition of a second hydrolase site to a serine esterase from the microbial communities inhabiting Lake Arreo, an evaporite karst lake in Spain [21]. The alternative binding site was found 15 Å from the main active site. The design reused existing Ser (residue 211) and acid (residue 25) residues, and only a Leu (residue 214) was mutated to His to configure the catalytic triad. The evolved pluriZyme, containing two catalytic sites, had boosted catalytic performance, substrate scope, stereoselectivity, and optimal temperature range. Moreover, the main active site of the evolved pluriZyme could be metamorphosed with the usage of covalent suicide inhibitors into a copper-based chemocatalytic site that catalyzed oxidation and Friedel–Crafts alkylation reactions [21]. This multifunctional catalyst allowed a one-pot cascade reaction involving esterase and oxidase activity: the conversion of 1-naphthyl acetate into 1,4-naphthoquinone and vinyl crotonate and benzene into 3-phenylbutyric acid.

The second pluriZyme was designed using a serine esterase from the same metagenomic origin as the first one but with a narrower substrate range [21]. The alternative binding site was found around 20 Å from the main active site. In this case, the design opted for the recycling of a present His residue (residue 34), and both the nucleophile and acid residues were added by mutating two Leu residues (residue 30 and 57, respectively). The new enzyme could catalyze 10 more substrates than the WT one, had better conversion rates, and had an increased optimal temperature window; additionally, the newly added active site could hydrolyze chiral molecules.

Next, the enzyme family was switched to an ω-transaminase, allowing cascade reactions of interest. The selected enzyme was a class III ω-transaminase from an acidic beach pool on Vulcano Island [43]. An ester binding site was found with PELE around 20 Å from the main transaminase site and turned into a hydrolase active site by reusing the acid residue (317) and adding the nucleophile and base residues by mutating an Ala (residue 172) and a Gln (residue 173), respectively. The new active site could hydrolyze around 50 ester substrates of an ester library of 96 compounds. Likewise, it was able to perform efficient transformations of different β-keto esters into β-amino acids.

More recently, we have explored adding other types of catalytic dyads/triads and, thus, chemistries by adding a Cys residue instead of a Ser. The first developed pluriZyme was used for this purpose, and the His residue of the artificial active site was reused to create a catalytic dyad by mutating a Leu (residue 24) to a Cys. This change granted protease activity to the serine esterase while preserving the other two active sites [44].

Finally, we have been working on adding feruloyl esterase activity in a xylanase. The xylan polymer contains ferulate radicals that slow down the depolymerization reaction, and adding specific feruloyl esterase- activity to the xylanase enzyme can speed up the reaction [45]. By inserting only two mutations, we were able to add a hydrolytic site with experimentally measured K_m of 2.8 $\pm$ 0.6 mM and V_{max} of 1641 $\pm$ 10 units/g against methyl ferulate, which is in the range of the best-performing esterases and lipases capable of degrading this substrate [46].

Some observations that we can extract from our experience working with this type of systems are regarding stability. In one transaminase design, for example, the second active site involved some residues located in a loop, introducing a significant stabilization effect (almost a 6-degree increase in the denaturation temperature) [22]. The fact that our mutants are designed to interact through hydrogen bonds might also explain the high success rate, helping the successful translation of the in silico predictions into the real in vitro assays. We have designed six pluriZymes, with six more to come. Only in one case did we not succeed in obtaining additional active sites: when aiming to introduce a second protease site in a small globular protease. The main reason for our failure seems to be the lack of an additional effective binding site(s); while some apparent sites were found, they did not represent a significant minimum in the substrate binding energy profile. Thus, the second step, the binding-site search, might be the bottleneck of the entire design process and a potential deal breaker.

In addition, one should consider that pluriZymes might introduce some drawbacks. Obviously, the first one is that the wild-type activity can be compromised. Moreover, the engineered artificial site has a tendency to be non-specific; additional engineering might be necessary to adapt it for specific substrates. In addition, the designed variants can introduce problems, although not frequent, with the expression/solubility or thermal (and even pH) stability. Finally, the catalytic efficiencies of many pluriZymes we have created are far from perfect (kcal/Km ~10^2–10^3 $M^{-1}{\cdot}s^{-1}$), with similar rates to artificial enzymes. These limitations show the difficulty of adding an efficient new active site to a functional enzyme without compromising the existing chemistry.

Table 1. Collection of all pluriZyme designs that have already been published. Note that we also add fraC, which is not a pluriZyme but was designed with the same methodology.

System (PDB ID)	Original Activity	Original Active Site(s)	Added Active Site	Improvement Fold [1]	Highest Activity [2]	ΔTm [3] (°C)	Topt [4] (°C)	PY [5] (mg/L)	Ref.
LAE6 (high-promiscuity esterase) [5JD4]	Esterase	S161/H286/D256	S211/L214H/E25D	average/max k_{cat}: ~3.4-fold/~74-fold	57.8 s^{-1} (phenyl propionate)	-	~35–45	12	[21]
LAE5 (low-promiscuity esterase) [5JD3]	Esterase	S15/H195/D192	L30S/H34/L57D	max k_{cat}: ~11.5-fold	6.28 U/mg (phenyl propionate)	-	~25	45.5	[21]
LAE6_pluriZyme [6I8F]	Esterase	S161/H286/D256 and S211/H214/D25	L24C/H214	-	2.63 U/mg (casein)	-	70–75	~10	[43]
class III ω-transaminase [7QYG]	Transaminase	K289	A172S/Q173H/E317	-	65.13 s^{-1} (phenyl propionate)	5.62	50–55 (transaminase activity) 60–65 (hydrolase activity)	32–45	[22]
Actinia fragacea porin [4TSY]	-	-	K20H/T21S	-	424 M^{-1} s^{-1}	2.1	35–45	15	[47]
Actinia fragacea porin [4TSY]	-	-	D38S/E173Q	-	1362 M^{-1} s^{-1}	6.3	35–45	30	[47]
Feruloyl esterase [8BBI]	Xylanase	E144/E251	*	-	1.64 ± 10 U/mg)	-	-	-	[46]

[1] Improvement fold refers to the improvement in activity when the sites share the same chemistry. [2] Highest activity means the most active substrate against the newly designed active site. [3] Difference in melting temperature. [4] Optimum temperature. [5] PY: protein yields. * This information is pending publication of the manuscript.

4. PluriZymes and Related Designs for Polymer Degradation

Since pluriZymes introduce hydrolytic triads, one would expect them to be good candidates for breaking polymers based on amide or ester bonds. In fact, one of the examples shown in the previous version, the LAE6 with the L24C mutation, is capable of degrading polypeptides (with an activity on casein equivalent to that of an industrial enzyme). Moreover, in the frame of the EU FuturEnzyme project (https://www.futurenzyme.eu/, accessed on 21 December 2023), we are currently working on adding additional hydrolytic sites to PETases.

The latest trends regarding bioplastic recycling seek to degrade polymers into their monomers and transform and reuse them in new materials in a process called bio-upcycling. This concept is currently being exploited, especially for polyethylene terephthalate (PET) [48], to promote the circular economy. The idea of applying pluriZymes to polymer biocatalysis is that different forms of enzymatic activity could be concatenated, enhancing the bioconversion process (Figure 4). In this way, having active sites with different catalytic activity/specificity can increase and modulate the overall activity so that some catalytic sites provide products that will be substrates for others.

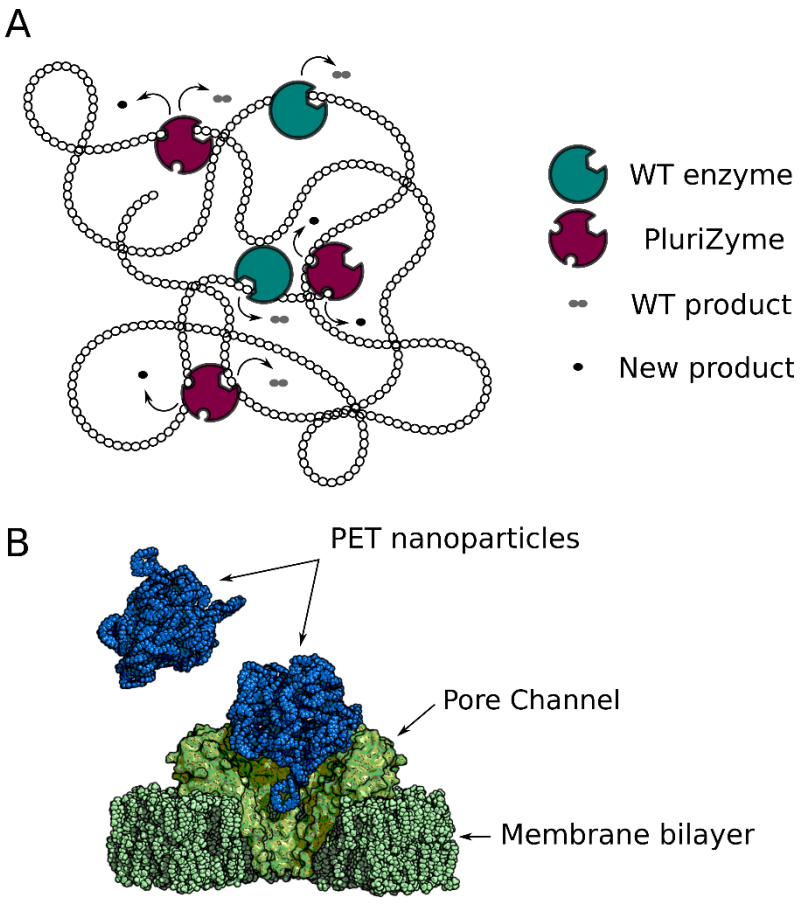

Figure 4. Example of a design for polymer degradation. (**A**) Concept of pluriZyme for polymer degradation. The addition of a new active site with different biochemistry to the WT enzyme scaffold can release new products of interest. (**B**) Graphic representation of FraC (PDB ID: 4TSY) nanopore (green) and PET nanoparticles (blue).

One example could be PET polymer. The products obtained from the enzymatic hydrolysis of PET are mainly ethylene glycol (EG), terephthalate acid (TPA), monohydroxyethyl terephthalate (MHET), and bis (hydroxyethyl)terephthalate (BHET). For example, BHET can be reused for PET production [49–51] or converted to quaternary ammonium compounds that can be used to soften textile cotton for industry [52]. TPA can be converted

into a wide range of aromatic compounds such as gallic acid, pyrogallol, catechol, muconic acid, and vanillic acid [53,54]. Moreover, TPA and its derivatives can be used to produce MIL-53 materials and organometallic derivatives [55]. Finally, EG can produce glycolic acid [56]. All these compounds are of industrial interest, and it would be attractive to couple the degradation process with a synthesis process in a coupled system of complex reactions.

Our results on building additional sites for PETases are still not satisfactory, possibly indicating the difficulty to establish and open a large enough site to bind and degrade PET. We have been significantly more successful, however, when following an alternative approach. The pluriZyme concept involves adding an additional active site to an enzyme, with the inherent difficulty of preserving, as much as possible, the original activity. Nevertheless, the workflow described in Section 2, capable of building catalytic triads, can be implemented in any protein. Thus, we turned our efforts to adding triads into a protein that could trap nanoparticles of PET efficiently. For this, we chose the homo-octamer membrane biological assembly crystal structure of *Fragaceatoxin C* (FraC, PDB ID 4TSY). We selected this protein scaffold for its pore dimensions, stability, and compartmental localization in membranes. We introduced esterase catalytic activity in two newly designed active sites and finally checked that they could be applied for PET nanoparticle degradation. The most active designed mutant located the triad in the alpha helix N-terminal domain, where non-conserved polar residues face the channel cavity of the pore. We rationally inserted two mutations, K20H and T21S, in the eight monomers, creating a new enzymatic complex with eight different potential active sites. In addition, the preexistence of two acidic residues, at positions 17 and 24, increased the number of possible combinations of catalytic triad formations. Thus, one can imagine that any nanoparticle (or nanoparticle protuberance, such as the one shown in Figure 4B that was obtained from molecular dynamics simulations) that is stacked in the pore will have eight pairs of scissors pointing at it and ready to cut the ester bonds. As a result, our FraC variant achieves one order of magnitude higher degradation rates than the current best PETases when operating on PET nanoparticles. In addition, our constructs hydrolyze multiple esters substrates: p-nitrophenyl acetate; p-NPP, p-nitrophenyl propionate; p-NPB, p-nitrophenyl butyrate, glyceryl tripropionate, phenyl acetate, and vinyl acetate [47].

5. Conclusions

PluriZymes seem to be a promising approach to make enzymes more appealing to the market. Adding a second active site can bring broader substrate specificity in hydrolases or a second biochemistry in a single protein scaffold, which could facilitate enzymatic one-pot cascade reactions. An obvious alternative approach includes recent successful attempts to fuse different proteins (see [20] and references therein), which might simplify the engineering efforts. PluriZymes, however, offer additional benefits, the first obvious one being that the technique reduces the overall protein cost. It mitigates expression costs and the necessity of optimizing different enzymes to harsh industrial conditions. Similarly, it helps in unifying operating conditions such as optimal pH and temperature. Moreover, it can ensure that both catalytic active sites are nearby, potentially accelerating the total reaction rate through substrate channeling [57–60]. Interestingly, most pluriZymes tend to be more stable than the native proteins. Mutations seek to introduce hydrogen bonds, which can easily introduce some additional stability. PluriZyme designs or similar approaches such as our recent success in turning a pore channel into a (very) efficient esterase, including PET nanopore degradation, open the door to exciting potential biotechnology applications. Imagine introducing dozens of pluriZymes into a unicellular organism to turn it into a specialized catalytic nanomachine. One might also think of using a la carte scaffolds, aiming at extreme operation conditions or ease of assembly into nanoparticles/nanodevices, and introducing efficient degradation active sites in them.

One other exciting concept is the usage of suicide inhibitors to change the type of reactions the functionalized site can perform [21]. As well as new inhibitor designs, we

could add more complex biochemistry; for example, we could use the pluriZyme design to join proteins through covalent (but reversible and controllable) linkers.

Of course, one can also imagine pluriZymes involving other catalytic constructs, in addition to hydrolytic triads (Figure 5). Significant attention has been already been paid to adding a metal site to allow redox reactions. This approach, widely studied in artificial metalloenzymes [61–64], could be a good starting point for other types of pluriZymes. For instance, inserting a superoxide dismutase metal site into an unspecific peroxygenase would allow the enzyme to be used without the need to add peroxide hydrogen, as it would be synthesized by the artificial site from superoxide radicals in the solvent. A second approach could involve adding amino-acid-based active sites, such as the archetypal glycoside hydrolases with two acidic residues acting as a dyad [64]. This could be useful in creating pluriZymes for degrading carbohydrate-based polymers. Another approach would be to use the same catalytic triad of a hydrolase (and thus the same computational design pipeline) but replace the catalytic Ser with an Ala residue, allowing us to catalyze aldol additions [65–67].

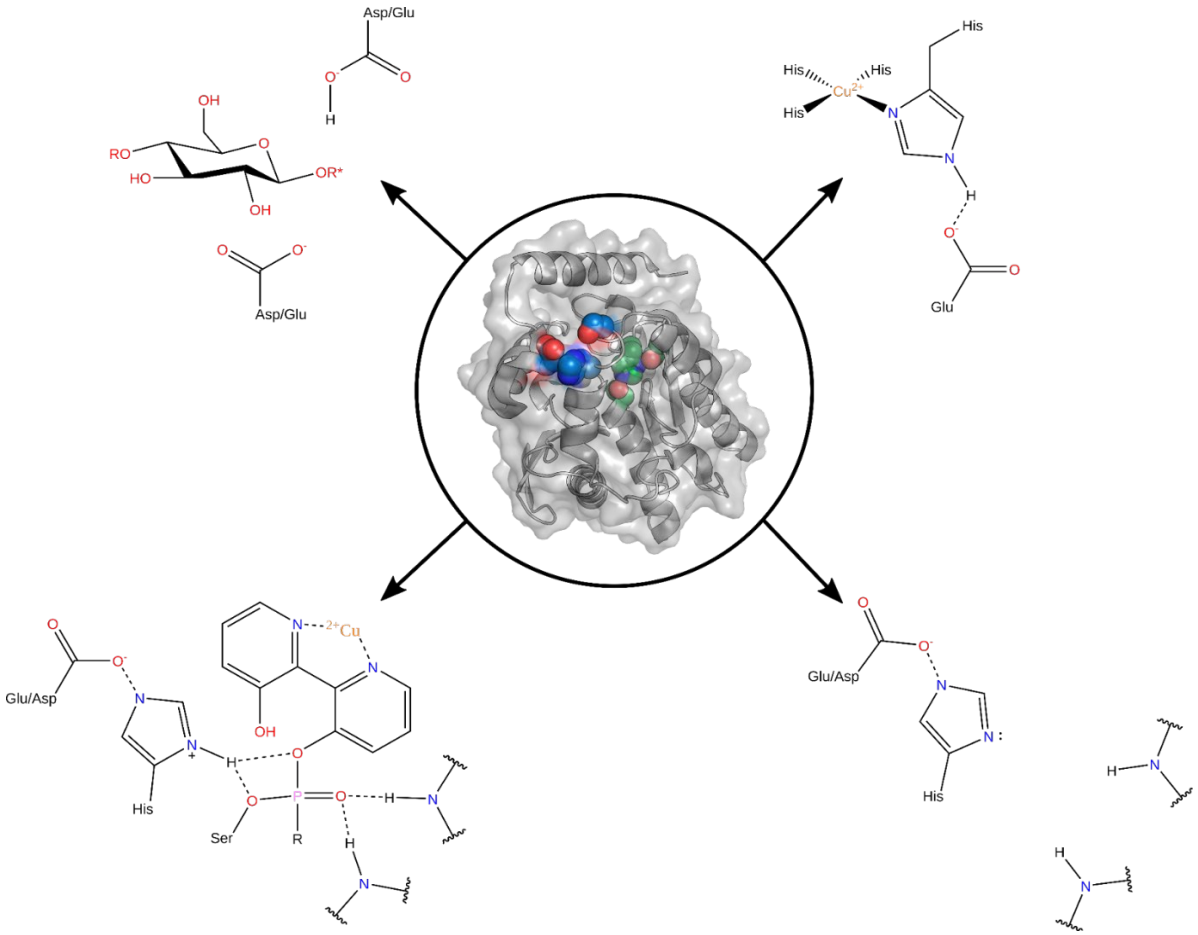

Figure 5. Different ideas to take pluriZymes to the next level. Adding a glycoside hydrolase site (**top left**). Using suicide inhibitors to change the chemistry of the added serine hydrolase site (**bottom left**). Mutating the catalytic Ser residue in a hydrolase site to allow aldol additions (**bottom right**). Designing a metal site, for example, a Cu−superoxide dismutase site (**top right**). The atoms in the depicted schemes are highlighted according to the element-based coloring from ChemDraw version 20.0.0.41. The asterisk on the top left means that the "side chain" (R) of the carbohydrate-like substrate could be different from the R at the other edge of the molecule.

Overall, we believe that pluriZymes are an exciting step that could significantly impact the functionalization of enzymes for biotechnological applications. With this review,

we attempted to provide a more exhaustive description of their design recipes to the community so that different laboratories can experiment with them and establish new limits. With modest computational resources and with help from our recipe, your first pluriZyme is one step closer.

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References

1. Lovelock, S.L.; Crawshaw, R.; Basler, S.; Levy, C.; Baker, D.; Hilvert, D.; Green, A.P. The road to fully programmable protein catalysis. *Nature* **2022**, *606*, 49–58. [CrossRef] [PubMed]
2. Wu, S.; Snajdrova, R.; Moore, J.C.; Baldenius, K.; Bornscheuer, U.T. Biocatalysis: Enzymatic Synthesis for Industrial Applications. *Angew. Chem. Int. Ed. Engl.* **2021**, *60*, 88–119. [CrossRef] [PubMed]
3. Arnal, G.; Anglade, J.; Gavalda, S.; Tournier, V.; Chabot, N.; Bornscheuer, U.T.; Weber, G.; Marty, A. Assessment of Four Engineered PET Degrading Enzymes Considering Large-Scale Industrial Applications. *ACS Catal.* **2023**, *13*, 13156–13166. [CrossRef] [PubMed]
4. Roda, S.; Santiago, G.; Guallar, V. Mapping enzyme-substrate interactions: Its potential to study the mechanism of enzymes. *Adv. Protein Chem. Struct. Biol.* **2020**, *122*, 1–31. [CrossRef] [PubMed]
5. Dauparas, J.; Anishchenko, I.; Bennett, N.; Bai, H.; Ragotte, R.J.; Milles, L.F.; Wicky, B.I.M.; Courbet, A.; de Haas, R.J.; Bethel, N.; et al. Robust deep learning–based protein sequence design using ProteinMPNN. *Science* **2022**, *378*, 49–56. [CrossRef] [PubMed]
6. Pavelka, A.; Sebestova, E.; Kozlikova, B.; Brezovsky, J.; Sochor, J.; Damborsky, J. CAVER: Algorithms for Analyzing Dynamics of Tunnels in Macromolecules. *IEEE/ACM Trans. Comput. Biol. Bioinform.* **2016**, *13*, 505–517. [CrossRef]
7. Richter, F.; Leaver-Fay, A.; Khare, S.D.; Bjelic, S.; Baker, D. De Novo Enzyme Design Using Rosetta3. *PLoS ONE* **2011**, *6*, e19230. [CrossRef]
8. Labrou, N. Random Mutagenesis Methods for In Vitro Directed Enzyme Evolution. *Curr. Protein Pept. Sci.* **2010**, *11*, 91–100. [CrossRef]
9. Agresti, J.J.; Antipov, E.; Abate, A.R.; Ahn, K.; Rowat, A.C.; Baret, J.-C.; Marquez, M.; Klibanov, A.M.; Griffiths, A.D.; Weitz, D.A. Ultrahigh-throughput screening in drop-based microfluidics for directed evolution. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 4004–4009. [CrossRef]
10. Jakočiūnas, T.; Pedersen, L.E.; Lis, A.V.; Jensen, M.K.; Keasling, J.D. CasPER, a method for directed evolution in genomic contexts using mutagenesis and CRISPR/Cas9. *Metab. Eng.* **2018**, *48*, 288–296. [CrossRef]
11. Anishchenko, I.; Pellock, S.J.; Chidyausiku, T.M.; Ramelot, T.A.; Ovchinnikov, S.; Hao, J.; Bafna, K.; Norn, C.; Kang, A.; Bera, A.K.; et al. De novo protein design by deep network hallucination. *Nature* **2021**, *600*, 547–552. [CrossRef] [PubMed]
12. Burke, A.J.; Lovelock, S.L.; Frese, A.; Crawshaw, R.; Ortmayer, M.; Dunstan, M.; Levy, C.; Green, A.P. Design and evolution of an enzyme with a non-canonical organocatalytic mechanism. *Nature* **2019**, *570*, 219–223. [CrossRef] [PubMed]
13. Lu, H.; Diaz, D.J.; Czarnecki, N.J.; Zhu, C.; Kim, W.; Shroff, R.; Acosta, D.J.; Alexander, B.R.; Cole, H.O.; Zhang, Y.; et al. Machine learning-aided engineering of hydrolases for PET depolymerization. *Nature* **2022**, *604*, 662–667. [CrossRef] [PubMed]
14. Mazurenko, S.; Prokop, Z.; Damborsky, J. Machine Learning in Enzyme Engineering. *ACS Catal.* **2020**, *10*, 1210–1223. [CrossRef]
15. Amrein, B.A.; Bauer, P.; Duarte, F.; Carlsson, J.; Naworyta, A.; Mowbray, S.L.; Widersten, M.; Kamerlin, S.C.L. Expanding the Catalytic Triad in Epoxide Hydrolases and Related Enzymes. *ACS Catal.* **2015**, *5*, 5702–5713. [CrossRef]

16. Hong, N.-S.; Petrović, D.; Lee, R.; Gryn'ova, G.; Purg, M.; Saunders, J.; Bauer, P.; Carr, P.D.; Lin, C.-Y.; Mabbitt, P.D.; et al. The Evolution of Multiple Active Site Configurations in a Designed Enzyme. *Nat. Commun.* **2018**, *9*, 3900. [CrossRef]
17. Petrović, D.; Risso, V.A.; Kamerlin, S.C.L.; Sanchez-Ruiz, J.M. Conformational dynamics and enzyme evolution. *J. R. Soc. Interface* **2018**, *15*, 20180330. [CrossRef]
18. Roda, S.; Robles-Martín, A.; Xiang, R.; Kazemi, M.; Guallar, V. Structural-Based Modeling in Protein Engineering. A Must Do. *J. Phys. Chem. B* **2021**, *125*, 6491–6500. [CrossRef]
19. Santiago, G.; Martínez-Martínez, M.; Alonso, S.; Bargiela, R.; Coscolín, C.; Golyshin, P.N.; Guallar, V.; Ferrer, M. Rational Engineering of Multiple Active Sites in an Ester Hydrolase. *Biochemistry* **2018**, *57*, 2245–2255. [CrossRef]
20. Carballares, D.; Morellon-Sterling, R.; Fernandez-Lafuente, R. Design of Artificial Enzymes Bearing Several Active Centers: New Trends, Opportunities and Problems. *Int. J. Mol. Sci.* **2022**, *23*, 5304. [CrossRef]
21. Alonso, S.; Santiago, G.; Cea-Rama, I.; Fernandez-Lopez, L.; Coscolín, C.; Modregger, J.; Ressmann, A.K.; Martínez-Martínez, M.; Marrero, H.; Bargiela, R.; et al. Genetically engineered proteins with two active sites for enhanced biocatalysis and synergistic chemo- and biocatalysis. *Nat. Catal.* **2020**, *3*, 319–328. [CrossRef]
22. Roda, S.; Fernandez-Lopez, L.; Benedens, M.; Bollinger, A.; Thies, S.; Schumacher, J.; Coscolín, C.; Kazemi, M.; Santiago, G.; Gertzen, C.G.W.; et al. A Plurizyme with Transaminase and Hydrolase Activity Catalyzes Cascade Reactions. *Angew. Chem. Int. Ed.* **2022**, *61*, e202207344. [CrossRef] [PubMed]
23. Díaz-Caballero, M.; Navarro, S.; Nuez-Martínez, M.; Peccati, F.; Rodríguez-Santiago, L.; Sodupe, M.; Teixidor, F.; Ventura, S. pH-Responsive Self-Assembly of Amyloid Fibrils for Dual Hydrolase-Oxidase Reactions. *ACS Catal.* **2021**, *11*, 595–607. [CrossRef]
24. Bos, J.; Browne, W.R.; Driessen, A.J.M.; Roelfes, G. Supramolecular Assembly of Artificial Metalloenzymes Based on the Dimeric Protein LmrR as Promiscuous Scaffold. *J. Am. Chem. Soc.* **2015**, *137*, 9796–9799. [CrossRef] [PubMed]
25. Zhou, Z.; Roelfes, G. Synergistic catalysis in an artificial enzyme by simultaneous action of two abiological catalytic sites. *Nat. Catal.* **2020**, *3*, 289–294. [CrossRef]
26. Palomo, J.M. Nanobiohybrids: A new concept for metal nanoparticles synthesis. *Chem. Commun.* **2019**, *55*, 9583–9589. [CrossRef] [PubMed]
27. Filice, M.; Marciello, M.; Morales, M.D.P.; Palomo, J.M. Synthesis of heterogeneous enzyme–metal nanoparticle biohybrids in aqueous media and their applications in C–C bond formation and tandem catalysis. *Chem. Commun.* **2013**, *49*, 6876–6878. [CrossRef] [PubMed]
28. Benavente, R.; Lopez-Tejedor, D.; Morales, M.D.P.; Perez-Rizquez, C.; Palomo, J.M. The enzyme-induced formation of iron hybrid nanostructures with different morphologies. *Nanoscale* **2020**, *12*, 12917–12927. [CrossRef]
29. Benavente, R.; Lopez-Tejedor, D.; Palomo, J.M. Synthesis of a superparamagnetic ultrathin $FeCO_3$ nanorods–enzyme bionanohybrid as a novel heterogeneous catalyst. *Chem. Commun.* **2018**, *54*, 6256–6259. [CrossRef]
30. Zhao, L.; Cai, J.; Li, Y.; Wei, J.; Duan, C. A host–guest approach to combining enzymatic and artificial catalysis for catalyzing biomimetic monooxygenation. *Nat. Commun.* **2020**, *11*, 2903. [CrossRef]
31. Jumper, J.; Evans, R.; Pritzel, A.; Green, T.; Figurnov, M.; Ronneberger, O.; Tunyasuvunakool, K.; Bates, R.; Žídek, A.; Potapenko, A.; et al. Highly accurate protein structure prediction with AlphaFold. *Nature* **2021**, *596*, 583–589. [CrossRef] [PubMed]
32. Sastry, G.M.; Adzhigirey, M.; Day, T.; Annabhimoju, R.; Sherman, W. Protein and ligand preparation: Parameters, protocols, and influence on virtual screening enrichments. *J. Comput. Aided Mol. Des.* **2013**, *27*, 221–234. [CrossRef] [PubMed]
33. Bochevarov, A.D.; Harder, E.; Hughes, T.F.; Greenwood, J.R.; Braden, D.A.; Philipp, D.M.; Rinaldo, D.; Halls, M.D.; Zhang, J.; Friesner, R.A. Jaguar: A high-performance quantum chemistry software program with strengths in life and materials sciences. *Int. J. Quantum Chem.* **2013**, *113*, 2110–2142. [CrossRef]
34. Acebes, S.; Fernandez-Fueyo, E.; Monza, E.; Lucas, M.F.; Almendral, D.; Ruiz-Dueñas, F.J.; Lund, H.; Martinez, A.T.; Guallar, V. Rational Enzyme Engineering Through Biophysical and Biochemical Modeling. *ACS Catal.* **2016**, *6*, 1624–1629. [CrossRef]
35. Lecina, D.; Gilabert, J.F.; Guallar, V. Adaptive simulations, towards interactive protein-ligand modeling. *Sci. Rep.* **2017**, *7*, 8466. [CrossRef] [PubMed]
36. Roda, S.; Fernandez-Lopez, L.; Cañadas, R.; Santiago, G.; Ferrer, M.; Guallar, V. Computationally Driven Rational Design of Substrate Promiscuity on Serine Ester Hydrolases. *ACS Catal.* **2021**, *11*, 3590–3601. [CrossRef]
37. Campbell, S.J.; Gold, N.D.; Jackson, R.M.; Westhead, D.R. Ligand binding: Functional site location, similarity and docking. *Curr. Opin. Struct. Biol.* **2003**, *13*, 389–395. [CrossRef]
38. Kua, J.; Zhang, Y.; McCammon, J.A. Studying Enzyme Binding Specificity in Acetylcholinesterase Using a Combined Molecular Dynamics and Multiple Docking Approach. *J. Am. Chem. Soc.* **2002**, *124*, 8260–8267. [CrossRef]
39. Rauer, C.; Sen, N.; Waman, V.P.; Abbasian, M.; Orengo, C.A. Computational approaches to predict protein functional families and functional sites. *Curr. Opin. Struct. Biol.* **2021**, *70*, 108–122. [CrossRef]
40. Pokkuluri, P.R.; Gu, M.; Cai, X.; Raffen, R.; Stevens, F.J.; Schiffer, M. Factors Contributing to Decreased Protein Stability When Aspartic Acid Residues Are in Beta-Sheet Regions. *Protein Sci.* **2002**, *11*, 1687–1694. [CrossRef]
41. Roda, S.; Terholsen, H.; Meyer, J.-R.-H.; Guallar, V.; Bornscheuer, U.; Kazemi, M. AsiteDesign: A semi-rational algorithm for automated enzyme design. *J. Phys. Chem. B* **2023**, *127*, 2661–2670. [CrossRef] [PubMed]
42. Chaudhury, S.; Lyskov, S.; Gray, J.J. PyRosetta: A script-based interface for implementing molecular modeling algorithms using Rosetta. *Bioinformatics* **2010**, *26*, 689–691. [CrossRef] [PubMed]

43. Coscolín, C.; Katzke, N.; García-Moyano, A.; Navarro-Fernández, J.; Almendral, D.; Martínez-Martínez, M.; Bollinger, A.; Bargiela, R.; Gertler, C.; Chernikova, T.N.; et al. Bioprospecting Reveals Class III ω-Transaminases Converting Bulky Ketones and Environmentally Relevant Polyamines. *Appl. Environ. Microbiol.* **2019**, *85*, e02404-18. [CrossRef] [PubMed]
44. Fernandez-Lopez, L.; Roda, S.; Gonzalez-Alfonso, J.L.; Plou, F.J.; Guallar, V.; Ferrer, M. Design and Characterization of In-One Protease-Esterase PluriZyme. *Int. J. Mol. Sci.* **2022**, *23*, 13337. [CrossRef] [PubMed]
45. Wang, W.; Zheng, D.; Zhang, Z.; Ye, H.; Cao, Q.; Zhang, C.; Dong, Z.; Feng, D.; Zuo, J. Efficacy of combination of endo-xylanase and xylan-debranching enzymes in improving cereal bran utilization in piglet diet. *Anim. Biosci.* **2022**, *35*, 1733–1743. [CrossRef] [PubMed]
46. Muñoz, R.; Guallar, V. *Rational Enzyme Engineering of Different Active Sites on a Xylanase*; Barcelona Supercomputing Center: Barcelona, Spain, 2022; pp. 66–67.
47. Robles-Martín, A.; Amigot-Sánchez, R.; Fernandez-Lopez, L.; Gonzalez-Alfonso, J.L.; Roda, S.; Alcolea-Rodriguez, V.; Heras-Márquez, D.; Almendral, D.; Coscolín, C.; Plou, F.J.; et al. Sub-Micro- and Nano-Sized Polyethylene Terephthalate Deconstruction with Engineered Protein Nanopores. *Nat. Catal.* **2023**, *6*, 1174–1185. [CrossRef]
48. Tiso, T.; Narancic, T.; Wei, R.; Pollet, E.; Beagan, N.; Schröder, K.; Honak, A.; Jiang, M.; Kenny, S.T.; Wierckx, N.; et al. Towards bio-upcycling of polyethylene terephthalate. *Metab. Eng.* **2021**, *66*, 167–178. [CrossRef]
49. Hu, Y.; Wang, Y.; Zhang, X.; Qian, J.; Xing, X.; Wang, X. Synthesis of poly(ethylene terephthalate) based on glycolysis of waste PET fiber. *J. Macromol. Sci. Part A* **2020**, *57*, 430–438. [CrossRef]
50. Köpnick, H.; Schmidt, M.; Brügging, W.; Rüter, J.; Kaminsky, W. Polyesters. In *Ullmann's Encyclopedia of Industrial Chemistry*; Wiley: Hoboken, NJ, USA, 2000; Available online: https://onlinelibrary.wiley.com/doi/10.1002/14356007.a21_227 (accessed on 12 October 2023).
51. Ji, L.N. Study on Preparation Process and Properties of Polyethylene Terephthalate (PET). In *Materials Science, Engineering, in Applied Mechanics and Materials*; Zurich Tomo: Zürich, Switzerland, 2013; Volume 312, p. 406. [CrossRef]
52. Shukla, S.; Harad, A.M.; Jawale, L.S. Recycling of waste PET into useful textile auxiliaries. *Waste Manag.* **2008**, *28*, 51–56. [CrossRef]
53. Liu, P.; Zheng, Y.; Yuan, Y.; Zhang, T.; Li, Q.; Liang, Q.; Su, T.; Qi, Q. Valorization of Polyethylene Terephthalate to Muconic Acid by Engineering *Pseudomonas putida*. *Int. J. Mol. Sci.* **2022**, *23*, 10997. [CrossRef]
54. Kim, H.T.; Kim, J.K.; Gil Cha, H.; Kang, M.J.; Lee, H.S.; Khang, T.U.; Yun, E.J.; Lee, D.-H.; Song, B.K.; Park, S.J.; et al. Biological Valorization of Poly(ethylene terephthalate) Monomers for Upcycling Waste PET. *ACS Sustain. Chem. Eng.* **2019**, *7*, 19396–19406. [CrossRef]
55. Ghasemzadeh, M.A.; Mirhosseini-Eshkevari, B.; Abdollahi-Basir, M.H. MIL-53(Fe) Metal-Organic Frameworks (MOFs) as an Efficient and Reusable Catalyst for the One-Pot Four-Component Synthesis of Pyrano[2,3-c]-pyrazoles. *Appl. Organomet. Chem.* **2019**, *33*, e4679. [CrossRef]
56. Zhan, Y.; Hou, W.; Li, G.; Shen, Y.; Zhang, Y.; Tang, Y. Oxidant-Free Transformation of Ethylene Glycol toward Glycolic Acid in Water. *ACS Sustain. Chem. Eng.* **2019**, *7*, 17559–17564. [CrossRef]
57. Poshyvailo, L.; von Lieres, E.; Kondrat, S. Does Metabolite Channeling Accelerate Enzyme-Catalyzed Cascade Reactions? *PLoS ONE* **2017**, *12*, e0172673. [CrossRef] [PubMed]
58. Gad, S.; Ayakar, S. Protein scaffolds: A tool for multi-enzyme assembly. *Biotechnol. Rep.* **2021**, *32*, e00670. [CrossRef] [PubMed]
59. Wheeldon, I.; Minteer, S.D.; Banta, S.; Barton, S.C.; Atanassov, P.; Sigman, M. Substrate channelling as an approach to cascade reactions. *Nat. Chem.* **2016**, *8*, 299–309. [CrossRef] [PubMed]
60. Aalbers, F.S.; Fraaije, M.W. Enzyme Fusions in Biocatalysis: Coupling Reactions by Pairing Enzymes. *Chembiochem* **2019**, *20*, 20–28. [CrossRef] [PubMed]
61. Davis, H.J.; Ward, T.R. Artificial Metalloenzymes: Challenges and Opportunities. *ACS Cent. Sci.* **2019**, *5*, 1120–1136. [CrossRef]
62. Schwizer, F.; Okamoto, Y.; Heinisch, T.; Gu, Y.; Pellizzoni, M.M.; Lebrun, V.; Reuter, R.; Köhler, V.; Lewis, J.C.; Ward, T.R. Artificial Metalloenzymes: Reaction Scope and Optimization Strategies. *Chem. Rev.* **2018**, *118*, 142–231. [CrossRef]
63. Wang, J.; Lisanza, S.; Juergens, D.; Tischer, D.; Watson, J.L.; Castro, K.M.; Ragotte, R.; Saragovi, A.; Milles, L.F.; Baek, M.; et al. Scaffolding protein functional sites using deep learning. *Science* **2022**, *377*, 387–394. [CrossRef]
64. Vornholt, T.; Christoffel, F.; Pellizzoni, M.M.; Panke, S.; Ward, T.R.; Jeschek, M. Systematic engineering of artificial metalloenzymes for new-to-nature reactions. *Sci. Adv.* **2021**, *7*, eabe4208. [CrossRef] [PubMed]
65. CAZypedia Consortium. Ten Years of CAZypedia: A Living Encyclopedia of Carbohydrate-Active Enzymes. *Glycobiology* **2018**, *28*, 3–8. [CrossRef] [PubMed]
66. Branneby, C.; Carlqvist, P.; Magnusson, A.; Hult, K.; Brinck, T.; Berglund, P. Carbon−Carbon Bonds by Hydrolytic Enzymes. *J. Am. Chem. Soc.* **2003**, *125*, 874–875. [CrossRef] [PubMed]
67. Rauwerdink, A.; Kazlauskas, R.J. How the Same Core Catalytic Machinery Catalyzes 17 Different Reactions: The Serine-Histidine-Aspartate Catalytic Triad of α/β-Hydrolase Fold Enzymes. *ACS Catal.* **2015**, *5*, 6153–6176. [CrossRef]