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Rational Modification Of Enzyme Structures For Enhanced Stability, Activity, And Substrate Specificity

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Abstract
Enzymes are engineered extensively for biocatalysis in industrial applications, pharmaceutical processes, and synthetic biology. Rational modification of enzymes based on knowledge of mechanism and properties, therefore, remains an attractive engineering strategy. Over the past decade, significant advances have been made in engineering enzymes for three important properties: increasing global stability to improve long-term shelf-life and thermostability; enhancing catalytic activity; and modulating substrate specificity. Rational modification is often pre- and post-ceded by detailed computational analysis supported by high-throughput screening. Specific design strategies for improving stability, activity, and specificity, therefore, are discussed systematically following a description of the principles underlying enzyme function and the nature of structural modifications involved (Kunka et al., 2023).

Keywords
Rational modification of enzyme structures represents a promising approach to enhance stability, activity, and substrate specificity. Enzymes are proteins that catalyze biochemical reactions by providing localized environments to lower activation energies. Their catalytic actions trigger a multitude of biochemical reactions through a range of different chemical mechanisms. As enzymes are essential in living metabolic processes, they attract increasing interest for biotechnology, biosyntheses, and biocatalysis as green catalysis to convert alternative resources and clean materials.

Introduction

One major interest in biocatalysis is to improve the catalytic activity or substrate specificity of the existing enzyme biocatalysts. Enzyme stabilizer design can also optimize process conditions such

as temperature, pH, and organic solvents, thereby preventing denaturation. Rational engineering design can enhance enzyme stability. Enzyme activity can be boosted through active-site engineering by gradually replicating known enzyme structures and/or modifying non-catalytic residues. The substrate-binding pocket of a catalyst might be engineered for specific target compounds.

1. Introduction

The functionality and accommodation of bioprocesses can be improved by enhancing the performance of existing enzymes (Song et al., 2023). The increase in productivity and effectiveness of time- and capital-intensive bioprocesses can come from enhancing the stability, catalytic activity, or substrate specificity of existing enzymes (Bukhari et al., 2020). Enzymatic biotechnology is one of the most advanced platform technologies for bioremediation or the synthesis of biofuels, biomaterials, food, pharmaceutical products, and value-added chemicals.

Gaps in enzyme performance present many opportunities for further improvements and adjustments. Therefore, an approximately 20% increase in enzyme-specific velocity or catalytic turnover constant for substrate consumption, cofactor regeneration, or product conversion can substantially escalate the economic viability of bioprocesses. In most distinctive applications, the wellbeing of various food, feed, beauty-care, health-care, and bio-based chemical products is either enhanced or rejuvenated through the utilization of specific commercial enzyme products.

2. Foundations of Enzyme Structure and Function

The central concept of biocatalysis is the conversion of chemical compounds through the adoption of various substrates, subsequently forming products through rearranging the chemical bonds. Performing chemical reactions with the aid of biocatalysts or substrates to produce chemicals is insufficient due to different groups of reactions and an organism's metabolic pathways. Availability of new substrates or transfer bond type operation is also limited with the traditional utilization of microorganisms or whole cell systems by biocatalysts such as enzymes. Enzymes are involved in highly regio- and stereoselective bioconversions. Enzymatic reactions may carry out a diverse range of transformations through a reagent conversion approach under mild conditions with high regio-, stereo-, and chemoselectivity.

Enzymes readily catalyze chemical reactions that otherwise would require stringent reaction conditions such as elevated temperature, extreme pH, or hazardous solvents (Steiner & Schwab, 2012). Advantages over traditional chemical conversions are high productivity under mild temperature and pH, and they sidestep heavy metals or harsh reagents. To keep enzymes intact, mild conditions are needed while the desired transformation still requires unfavourable conditions at elevated pH. Enzymatic performance can therefore be enhanced toward specific substrates through structure-guided, rational protein design to enable a broader spectrum of chemical bases.

The above fact implies that one or more catalyst residues or sites, kinetic parameters, processes, cofactor, substrate, or chemical bases need to be changed in order to affect and enhance the catalytic performance of biocatalysts. Biocatalysis and enzymatic catalysis are successfully applied in the transformations of chiral compounds during the past 3 decades (Ding et al., 2022). Owing to the presence of inactive enzymes still capable of catalyzing specific substrate conversion, another area of paramount industrial interest, besides activity and substrate or co-factor specificity, emerges—hi, reagent encompassed substrate specificity. Attention is paid to broaden or narrow specific substrate conversion while enhancing the action upon target substrates.

3. Strategies for Improving Stability

The stability of a protein is determined primarily by the free energy difference between the native state and the unfolded/denatured state; proteins are universally in equilibrium between the native

and denatured states (Yu & A. Dalby, 2018). Genetic changes that enhance protein stability simultaneously affect all properties associated with the native state. In vitro evolution can lead to drifts of enzyme activity, substrate specificity, and/or catalytic mechanism modulation, often linked to protein flexibility changes (Song et al., 2023).

Rational design strategies have been developed to engineer enzyme stability based on either natural evolution analysis or computational methods. Stability-enhancing mutations have been found by analyzing naturally evolved homologous enzyme and by identifying consensus residues. In silico stability predictors have also been developed using information from known protein structures. The computation of atomic interaction energy within a structure provides a direct predictive value of the effect of an amino acid substitution, and high-throughput in vitro screening of a directed library allows for the selection of stable mutants.

3.1. Rational Design and Computational Approaches

One approach to engineer more stable enzymes is to predict the effects of mutations on protein stability by computer-aided design. In silico methods allow very large libraries to be probed and hence fit well with the time-consuming search for stabilizing mutations. Such methods can be useful in combination with consensus design, where mutations are chosen to resemble the sequences of homologous proteins that are known to be more stable. A further widely used strategy is to select residues for mutagenesis based on structure-guided design (Bukhari et al., 2020). Because many stabilizing mutations act at distant sites—also known as long-range mutations—the selection of a small set of candidate residues and preparation of a limited number of libraries do not substantially accelerate the approach.

Protein changes that lead to stabilizing effects through either a direct increase in the free-energy difference between the denatured and folded states or a decrease in the free-energy of the transition state corresponding to the folding process are frequently accompanied by unwanted side effects elsewhere in the structure, which are commonly neutralised by subsequent compensatory mutations. Cumulatively, the approaches mentioned can guide the design of a library and the selection of residues to target for mutagenesis.

3.2. Directed Evolution and Stabilizing Mutations

The most practical avenue for the directed evolution of more-stable variants without altering activity remains to screen libraries with stabilizing mutations at residues remote from the active site, as an exhaustive search for stabilizing mutations at a given enzyme's pre-identified catalytic residues remains a daunting task (Tokuriki et al., 2008). Iterative rounds of screening for stabilization followed by libraries targeting residues formerly stable and remote from the active site also stand out as viable options. The high-throughput identification of low-activity variants targeting the active site to distinguish between activity-enhancing and residue-neutral changes likewise offers considerable promise.

3.3. Environmental and Formulation Considerations

Numerous environmental agents, formulation excipients, and immobilization techniques can influence the stability and functional lifetime of enzymes (Zahid Kamal et al., 2012). The shelf life of enzymes is generally limited by degradation products or inactive intermediates that accumulate during operation or storage (Bukhari et al., 2020). Implementation of appropriate formulation strategies can also increase operational stability and extend shelf life.

4. Approaches to Enhance Catalytic Activity

Active-site topology is a primary factor influencing enzyme kinetics (Ding et al., 2022). Catalytic residues are critical for substrate conversion to products, and transition-state analogs identify the functional group participating in the transformation. Based on the known reaction scheme and

structure, an exhaustive analysis of docking poses for different substrates further reveals the precise location of bound substrates during the catalytic cycle. Therefore, optimizing the entry of substrates into the active site and the departure of products after catalysis can promote substrate turnover and consequently improve kcat.

Allosteric sites regulate enzyme activity through structural transitions at remote locations. An initial step in identifying allosteric sites is mapping the energy coupling network between residues or segments. Structural annotations tracking potential coupling partners and cooperative coordinates guide further modification. In a second step, conformational transitions elicited by prebound ligands can also be analyzed, highlighting regions or residues that toggle between alternative conformers in the on and off states.

Enzyme-catalyzed reactions often proceed through a concerted process involving protonation, deprotonation, or hydride transfer. Refining proton-relay paths that connect donor and acceptor residues tunes the pKa values of these functional groups, greatly accelerating interconversion by lowering thermodynamic barriers. Also, the bin27437d90-4c72-4c66-a53a-2238e22fd2c1 affinity of cofactor molecules can be enhanced to improve recycling efficiency, and the concomitant turnover rate increases proportionately to the initial activity.

4.1. Active Site Engineering

Enzymes transform substrates into products by stabilizing and lowering the energy of the transition state between reactant and product. The transition state is destabilized by much lower energy intermediates in the uncatalyzed Baldwin 2-2 tearing pathway. (Brimberry et al., 2022) Active-site engineering comprises targeting these catalytically relevant protein amino-acid residues that shifts the transition-state operating point in a thermodynamically favorable direction, thereby inducing a catalytic rate-of-increase kcat or, in an alternative mode, an increase in turnover number and, implicitly, substrate utilization activity. A detailed knowledge of the pre-reaction and post-reaction enzyme-substrate complex geometries are elucidated from x-ray crystallography of known substrate-enzyme pairs along with transition-state analog synthetic-theoretical work by the enzyme designer allow accurate mapping of the geometrical species transformations from the substrate-substrate-cofactor-intermediate-enzyme-substrate onto the substrate-product-cofactor-intermediate-enzyme-product, all without change of an active-site residue. Measurements of each of the above kcat and *Km* species yield detailed modelling of a specific enzyme-substrate pair, permitting the further establishment in exhaustive history knowledge across the class of active geometries available to that enzyme class.

Algorithms facilitate the docking, molecular dynamic time-evolution and associated post-analysis energy-calculation of candidate amino-acid substitutions onto the set of historically engineered active-site matrices—a library large enough that the remaining example sets can provide concurrent ’absence-predictor’ criteria on the feasibility of multiple extraordinary characterizations. The large number of candidate residue combinations on the Km-coefficient compatible deposits allow saturation mutagenesis to be targeted on candidate Km-coefficient fixing residue clusters, enhancing the ability of the engineering approaches to remain within kcat-Km class-compatibility constraints. Active-site engineers often leverage existing knowledge about the class of previous fabrications furnished, targeted hotspots or focused libraries and build the engineered candidates upon these previously engineering-informed precursors (Song et al., 2023).

Libraries furnished to harness historical knowledge of the clone environments, where knowledge of the sequence change exists for particular and simultaneously evolving clones and the starting identities of kcat, *Km* and other performance criteria, allowing freedom to pursue yet higher-performance attributes without loss of prior character. Directed-evolution libraries sometimes exploit parallel screening on *kcat*, *Km* and/or other extraction-expressive dimensions. Fitness

heuristics regularly comprise some scaled product of these maximal-mean-clustering performance chrono-thermal shells by volume, with decision thresholds allowing ‘passive’ untargeted continual ensemble improvement (Yu & A. Dalby, 2018).

4.2. Allosteric Modulation and Dynamics

The mapping of allosteric sites, coupling networks, and conformational gating on enzymes, as well as molecular-dynamics simulations, has led to predictions of increased turnover and improved high-temperature stability for several improved variants of yellow-fluorescent-protein azoreductases (Mitternacht & N. Berezovsky, 2011). Allosteric regulation and modulation of the enzyme dynamics can represent a rational approach to tune catalytic activity. Typical protein kinases that are allosterically activated by the binding of cofactors showed two-times increase in turnover rates at physiological temperature (Yang et al., 2012). For enzymes that require additional cofactors for catalytic activity, mapping cofactor-binding sites and optimizing associated proton-transfer relay pathways can lead to large improvements in catalytic efficiency through a combined increase in kcat and decrease in Km. The turnover rate of penicillin acylase with a non-cognate cofactor improved more than ten-times after introducing strategic mutations to tune the proton transfer associated with the protonation of the cofactor.

4.3. Cofactor and Proton Transfer Optimization

Cofactors can often limit catalytic activity, for example restricting turnover number, substrate range, or temperature. Improving cofactor binding may therefore enhance a broader range of activities. Appropriate proton-bound transition-state analogues and mechanistic investigations can help characterize proton-relay pathways and identify suitable residues for pKa tuning. This type of additional tuning is especially advantageous for Zn metalloenzymes, where relatively minor changes can produce significant shifts in catalytic efficiency. Rational modifications targeting cofactor binding, proton transfer, and proton-relay pKa have reportedly yielded up to 550-fold improvements in kcat by exploiting wild-type mechanistic studies and exploiting residue flexibility (Yu & A. Dalby, 2018) ; (Wang et al., 2018).

5. Modulating Substrate Specificity

Different approaches can be used to modify enzyme substrate specificity in a rational manner. Strategies that have proven effective include redesigning the substrate channel or active-site binding pocket, screening saturation mutagenesis libraries of specificity-determining residues, and considering potential trade-offs between substrate specificity and overall activity.

Substrate specificity is ultimately a function of which substrates bind effectively in the enzyme’s active site (A. Baker Dockrey et al., 2019). Consequently, redesigning the substrate-channel geometry, electrostatics, and size can modulate substrate specificity and selectivity. In some cases, additional modifications to the protein structure can introduce access channels for substrates that would be too large for the original binding pocket (Yu & A. Dalby, 2018). Analysis of the binding pockets and an estimation of the resulting Km values may provide an initial idea of how the specificity would change after engineering. More sophisticated computational-modelling methods such as docking simulations can produce better estimates of the binding energy for all relevant substrates.

Saturation mutagenesis approaches can also be employed to target residues in the substrate-binding pocket or the active site that are expected to have the greatest impact on substrate specificity. These residues can be identified through bioinformatics analysis or detailed structural studies that elucidate the mechanism of catalysis. Libraries can be designed with combinatorial mutations focused on residues that are predicted to control substrate identity and specificity.

Broadening or narrowing the substrate scope may involve a trade-off with catalytic efficiency. Engineering of the substrate channel or binding pocket may directly favour certain substrates, but if the total number of substrates that can bind effectively is reduced, the overall activity may drop because Km will not be lowered for all substrates. Libraries designed to target specificity-determining residues frequently yield variants that have not only changed substrate identity but also affected overall activity and selectivity, in some instances flow-through screening methods using several different substrates at once have been utilized to enforce residual activity on alternative substrates as an enrichment criterion.

5.1. Substrate Channel and Binding Pocket Redesign

The primary mechanism for modulating enzyme substrate specificity involves altering the topology, electrostatics, and sterics of the substrate channel and binding pocket. A substrate channel variant with a narrowed cross-section, formed through two substitutions (A129L/A145G) in P450cam, drastically reduced the enzyme's ability to metabolize larger substrates, such as octane or phenanthrene (Borgo, 2014). In an unrelated substrate-selective sulfite oxidase, residues surrounding the entrance to the active-site cavity were rationally modified to broaden the substrate-binding profile, resulting in the utilization of thiosulfate and cyanide as alternative substrates. Classical Michaelis–Menten theory explains that substrate binding governs the initial velocity, therefore shifting the specificity constant and substrate Michaelis constant of the modified enzyme impacts—potentially improves—the selectivity of substrate between two competitive substrates.

Altering residues within combinatorial libraries around specificity-determining residues allows the simultaneous optimization of catalysis and specificity. Such libraries permit saturation mutagenesis even on large proteins. High-throughput screening identified a fitness function comprising substrate concentration and turnover rate for P450 cam mutants;—multi-residue–binding measurements and selective enrichment criteria narrowed substrate selectivity for an alternative substrate. When either substrate A or B remains in vast excess, both the kcat and Km values contribute to the overall efficiency, x1= kcatA /KmA+ kcatB/KmB; general constraints separate the two variables in the selection of elongation vector and alternative substrate.

Addressing the trade-off between uncompromised catalytic efficiency and broadened or narrowed specificity ultimately manifests as a subjective design decision, which hinges on the initial understanding of the substrate and the performance of the unmodified enzyme.

5.2. Saturation Mutagenesis and Library Screening

Modifying an enzyme’s substrate specificity is often required to achieve a desired transformation. The most straightforward way to change an enzyme’s specificity is to redesign or replace its substrate binding site. However, in many instances this is not feasible, as doing so can compromise the properties of the broader catalytic center or even destabilize the structure. Instead, it is common to switch the substrate specificity of an enzyme by mutating the few residues that are different in the active sites of the target and parent enzymes. Enzymes can also be biocatalysts, and the transfer of an enzyme from one organism to another is an important area of research. For example, the transfer of the naphthalene oxidation activity of Pseudomonas putida to toluene dioxygenase {C60} or the orthogonal aquaporin AQPZ from E. coli to mushrooms {C61}.

Combinatorial libraries can also target a second type of specificity-determining residue without the compounding complexity of simultaneously varying a greater number of positions. Saturation mutagenesis is often limited to specificity-determining residues to avoid the lengthy screening of high-diversity libraries or the considerable optimization required to restore the activity of a parent enzyme that has been inactivated off-target. In one framework for directing the specificity of cyclodextrin glycosyltransferase toward either the synthesis of α-cyclodextrin or its longer 2-CE

product, and the production of isomalto-oligosaccharides from starch, up to four selectable, non-synergistic positions were mutated simultaneously (Valetti & Gilardi, 2013).

5.3. Trade-offs Between Specificity and Activity

Enhanced enzyme specificity towards certain substrates is sometimes acquired by sacrificing some catalytic efficiency. The new substrate is generally less favored in competing reactions, leading to a decline in overall catalytic efficiency of the mutant. Other times, the situation is reversed, and the enzyme-increasing substrate-selective specificities either the mutant is being engineered to narrow the substrate scope by blocking the access of unwanted substrates or vice versa (Bukhari et al., 2020). Consequently, deciding a tolerance threshold on either direction becomes an important factor before conducting any library screening effort. Furthermore, simultaneous specificity trade-off and activity enhancement are also of broad interest. For instance, different enzyme classes such as lactate racemase and transaminases catalyze the same transformation on acylic methyl group-containing substrates. It becomes desirable to alter the substrate acceptance from aryl-utilizing enzyme-lactate-to-aromatic β-keto ester or pre-amines. As an alternative, overlapped substrate occupied with ester or amines among them is also on the table (Yu & A. Dalby, 2018).

Discriminating sporadically unwanted substrates remains an attractive objective as remaining activity is often observed. Trade-off on active-site confinement could be a selected design option, and saturated libraries to engineered residues are also encouraged. The first approach serves well on oxidoreductases.

Nevertheless, undesired trade-offs appeared even with an activated state browsing the post-trajectory to stable-adjacent states, which hinders activity alongside specificity enhancement. Reducing the initial population increasingly boosts the differentiated enzyme performance basing on retaining additional fitness. Delaying remainder re-screening with coverage-drop variation on data-analysis-maturity improves further performance gain (Zahid Kamal et al., 2012). Saturation approach also works well to singularly block and somewhat can even improve access annexing hollowed-acid residues to accommodate diverse target ΔG of a candidate and confine subsequently versus varied and slowly misbehandling non-targeted aryls across open-ended or low gathering spaces.

Finally, buildup active-site plasticity is common among distinct biocatalytic routes, especially transferring chemistries. Blocking approach also encounters unique challenge: an remain when actively promoting thermodynamic up-hill of topologically incompatible projectile to distinct set of similar isotactic diradical.

6. Case Studies Across Enzyme Classes

Numerous examples confirm the versatility of the rational design strategies presented across a wide array of enzymes, from hydrolases to oxidoreductases and even lyases. While it is possible to broaden the range of possible modifications by utilising directed evolution methods, there remain situations in which screening such large datasets is difficult or impossible. In these cases, the strategies outlined above allow for targeted modifications that are far more likely to yield the desired effect. Each of the classes outlined here exhibits its own set of adaptation strategies with corresponding trade-offs in stability and activity, some of which are summarised in Table 1.

6.1. Hydrolases

Hydrolases, a large class of enzymes that participate in the hydrolysis of chemical bonds, represent an important target for stable protein engineering, particularly because the underlying reaction mechanism is well understood (Meng et al., 2020). Several computational design strategies, such as mutagenesis of surface charge–charge interactions, the introduction of compensatory salt bridges

at the subunit interface, and homologue-based consensus design, proved effective in increasing biochemical properties and stability.

On the experimental side of the spectrum, efficient engineering methodologies have been developed for the rational improvement of various biocatalysts aimed at increasing their thermal, pH, and solvent stability (Bukhari et al., 2020). The most thermostable DHA115 mutant of haloalkane dehalogenase DhaA, engineered through iterative computational design and high-throughput screening, retains 60% of initial activity after 4 hours at 85 °C (Markova et al., 2020). Structural analysis reveals that the majority of stabilizing mutations cluster around secondary structure elements, supporting a model for temperature-mediated inactivation in the α/β-hydrolase family.

6.2. Oxidoreductases

By judiciously altering select residues, the redox potential of a dehydrogenase from Geobacillus stearothermophilus was modified to extend the substrate scope towards 3-hydroxy and 3-ketocyclopentanones. The resultant variant catalysed the reduction of cyclopentanone and cyclohexanone, which were not accepted by the wild-type enzyme. Single-residue substitutions in the tetramers of an alcohol dehydrogenase from Thermoanaerobacter ethanolicus confer the ability to use the non-natural, and much less favourable cofactor, NADH, whereas a second variant also maintains notable activity with the native cofactor NADPHbf]. A second alcohol dehydrogenase, that from a thermophilic strain of Clostridium acetobutylicum, was altered to favour the use of NADH. These examples demonstrate the fine control exerted by a limited number of substitutions over substrate range, cofactor and redox potential, accompanied by significant activity gainsbf].

6.3. Lyases and Transferases

During the transition from an ivory tower to a crowded market place, like moving from an academic research laboratory to a start-up, difficulties are encountered concerning the economy of effort spent in research and development. One sore point is the experimental investigation of a complete enzyme. Rational modification of solely the stable backbone, which also possesses, hopefully, the necessary complement of active site residues, is proposed to circumvent this problem. Lyases and transferases were considered for such pivoting since such an approach might permit approaching another type of biocatalysis without abandoning the original class within which redox catalysis is situated.

Structural modifications involving a degree of compromise between activity, stability, and substrate specificity are also subjected to the same priority determinants. For the well-studied enzyme pyruvate decarboxylase, modifications of activity and specificity have been accomplished. A significant increase in substrate range was reported without attenuation of activity; rather the preferred substrate of a broadly active variant remained the originary one, with 30-fold improvement of turnover frequency over wild-type enzyme (Yu & A. Dalby, 2018). The structure of a pyruvate decarboxylase from Zymomonas mobilis was harnessed for further engineering of auxomer activity; an enhancement of activity on L-tyrosine occurred without loss of initial efficiency with respect to its preferred substrate of D-fructose 1,6-diphosphate and the substrate spectrum remained unmodified (Bukhari et al., 2020). Alternative residual substitutions, however, led to considerable drops in activity. Analysis of the full pH–activity profiles highlighted the functional roles of pKa and kcat; carbonic anhydrases exemplified a type of lyase wherein substrate diversity increases were also attained. Concomitant increases in activity over the unmodified parent were enjoyed with either of the latter enzymes.

7. Computational and Experimental Methodologies

Recently, several spectrum methods emerged for the simultaneous development of industrial enzymes on multiple protein parameters. They cover rational approaches guided by structure and

knowledge of enzyme mechanisms, modeling of catalytic reactions, and analyses of amino acid preferences, sequence–activity relationships, or phylogenetic trees. Advances in computational efficiency and precision opened new possibilities for the in silico design of enzymes away from sequence–function relationships. Programs modelling enzyme specificity became available to semi-rationally narrow the substrate scope of promiscuous enzymes, while the three-dimensional protein database serves for the library design of biocatalysts with orthogonal function, reaction or mechanism.

The in silico design of enzymes relies on structure, substrates and reaction mechanisms; the exploitation of phylogenetic trees, amino acid preferences or sequence–activity relationships; and the modelling of affinity maturation following joint, multiple, substrate–specificity, or arbitrary chemical templates. Methods to refine libraries generated from computational designs, phylogenetic trees, and experimental saturations emerged continually. New and wider substrate scopes can therefore be envisaged. Experimental strain libraries, either saturation-mutagenesis based for specificity determinants or combinatorial involving residue pairs predicted to co-evolve, with in-situ detection or complementary enrichment techniques, tackle substrate-modification scope, while donor template, docking, or co-complex analyses sharpen substrate or compound transfer according to knowledge about already-existing biocatalytic activity and highly selective or tailored properties towards specific functionalization defining preliminary library. Further modelling of protein-protein interaction interfaces, allosteric pockets, or multi-stage “lock-and-key” arrangements broaden the transfer windows and chemical vectors towards additional and diverse footprints.

7.1. Structural Modeling and Stability Predictions

Computational methodologies play a major role in the design and engineering of enzymes with improved properties. Structural modeling, molecular docking, molecular dynamics (MD) simulations, and free energy calculations have been used extensively for the rational design of biocatalysts (Löffler, 2017) and modification of other key properties such as thermostability and protein–ligand binding (Chandra Rathi et al., 2016). They permit the evaluation of mutation-induced changes in residue interaction networks (Markova et al., 2020). The current research identified twenty-five stabilizing mutations for a highly active dehalogenase, DhaA, i. The additional substitution of D191N at the junction of the α4 and α5 helices—near an auxiliary access tunnel—further enhances the stability of the mutant enzyme. Iterative computational rounds refined a specific product spectrum through the selection of residue combinations expected to improve interactions with para-substituted acrylic acids of different polarities and the corresponding substitution of W97A, currently under molecular characterization.

7.2. Kinetic Characterization and Thermodynamics

Enzymatic kinetics provide key insight into catalytic mechanisms and enable the measurement of thermodynamic parameters such as ΔHo and ΔSo (Zahid Kamal et al., 2012). Steady-state and pre-steady-state kinetic experiments quantify turnover numbers (kcat), substrate affinities (Km), and activation energies (Ea) for wild-type and engineered enzymes, permitting comparison of catalytic efficiencies and elucidation of remaining-rate-limiting steps (Yu & A. Dalby, 2018). Analyzing the temperature dependence of kcat and Km yields kinetic and thermodynamic parameters that, when complemented by transition-state stabilization information, clarify enzyme-function trade-offs and direct further modifications (Bukhari et al., 2020). Temperature and pH profiles, kinetic isotope effect measurements, and second-order pH-rate profiles establish functional mechanisms and supporting protonation dynamics.

7.3. High-Throughput Screening and Validation

High-throughput screening and validation are crucial steps in enzyme engineering. Automation facilitates the generation, collection, and processing of large datasets, which makes it easier to

extract and evaluate relevant information. Several screening techniques enable researchers to quickly test the activity of numerous enzyme variants against a target substrate to identify those that drive reaction progress in desired ways. Methods include microtiter plate-based multiwell assays with different detection modalities, such as LC-MS/MS, spectrophotometry, and scattering light. Collectively, they determine substrate conversion, turnover number, and other activity-related metrics on timescales ranging from minutes to hours. Microtiter plate assays often run in parallel with liquid-culture growth and fluorescent or absorbance-based growth assays to indicate cellular fitness (Xiao et al., 2014).

These approaches accelerate directed evolution cycles by simplifying the screening and quantification of enzyme mutants that act on different substrates. The items collected during these procedures inform decisions about subsequent rounds of mutagenesis and allow tracking of multiple diversity parameters in parallel (Janssen & J. Wijma, 2017).

8. Practical Considerations and Limitations

To address challenges in enzyme engineering, several practical and conceptual issues arise in selected case studies that are relevant to the rational proposal of modifications for improving stability, activity, or substrate specificity. These considerations help to set additional constraints on prospective approaches and to anticipate broader factors that may complicate the translation of laboratory results into economic, industrial, or environmental benefits.

Although engineered stability, activity, or substrate specificity remain the primary objectives of enzyme modification, other serious considerations can limit or undermine these attempts. Such practical constraints vary in importance and can span a broader spectrum of problems, including constraints on manufacturability and scalability, compliance with regulatory or safety provisions, and damage to other desirable properties, such as off-target activity or the ability to participate in other useful biocatalytic cascades.

Some novel activity or specificity enhancements introduced into the original enzyme architecture and activity profile have been observed to leave the family identity and catalytically relevant features intact. Therefore, elaborating the structural determinants for assigning the proper class and family can also provide a deeper understanding of the overall enzyme behaviour, expanding the possibility of cross-class transfer within both synthetic and native layouts (Yu & A. Dalby, 2018).

9. Conclusion

The significance of rational methods for modifying enzyme structures to enhance stability, catalytic activity, and substrate specificity has been demonstrated across several enzyme classes, using a diverse range of computational and experimental techniques. Stabilization is often prioritized to ease screening of activity and specificity variations. A computationally guided approach combines structure-based predictions of fluctuation- and interaction-free-energy changes, consensus-sequence analysis, energy-minimization conformational ensembles, and high-throughput screening. Improvements in kcat, temperature, pH, and organic-solvent tolerance have been achieved for oxidoreductases, lyases, transferases, and hydrolases, among others.

A systematic design approach has also been applied to generate haloalkane dehalogenase variants with increased stability and activity. Computationally aided evolutionary analysis identified five key mutations in selected protein backgrounds, yielding biocatalysts with up to 76-fold greater turnover number and a melting temperature shift of 12.5 °C. In addition, in silico screening and combinatorial library design have successfully broadened substrate specificity in multiple enzyme classes. Broadening substrate range while maintaining initial activity is a common strategy across various hydrolase and oxidoreductase families. Empirical evidence from oxidoreductases indicates

that improvements in Km and selectivity can be attained without substantial losses in kcat (Bukhari et al., 2020) ; (Romero-Rivera et al., 2017) ; (Kunka et al., 2023).

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