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Web-based tools for computational enzyme design

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Enzymes are in high demand for very diverse biotechnological applications. However, natural biocatalysts often need to be engineered for fine-tuning their properties towards the end applications, such as the activity, selectivity, stability to temperature or co-solvents, and solubility. Computational methods are increasingly used in this task, providing predictions that narrow down the space of possible mutations significantly and can enormously reduce the experimental burden. Many computational tools are available as web-based platforms, making them accessible to non-expert users. These platforms are typically user-friendly, contain walk-throughs, and do not require deep expertise and installations. Here we describe some of the most recent outstanding web-tools for enzyme engineering and formulate future perspectives in this field.

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Introduction

Enzymes are the catalysts used by nature to perform the complex chemical reactions required to sustain life. They evolved over billions of years to achieve the high efficiency and specificity needed for each lifeform to survive and thrive in their environments. Biotechnology has emerged as a way for mankind to exploit such nature's creations, with numerous benefits over the classical chemical processes. In many cases, however, technological applications require particular properties beyond what is available in naturally occurring biomolecules, such as specific activity, selectivity, stability, solubility, etc. In such cases, they have to be reengineered on-demand [1].

The global protein-engineering market was evaluated in USD 1.9 billion in 2018, and it is projected to reach USD 3.9 billion in 2024 with a remarkable annual growth of 12.4% (CAGR) during this period. The rational protein design accounted for the largest technology segment of the market in 2018, while the biopharmaceutical companies accounted for the largest end-user [2]. This categorically demonstrates the growing importance of protein engineering.

Directed evolution methods have been extensively used to improve natural biomolecules successfully. However, they can be expensive and time-consuming. Therefore, the usage of computational methods for rational design is becoming more and more common. The predictive power of computational tools is gradually improving. Many of the existing tools are developing intuitive and user-friendly web-based platforms, which expand their usability to the broader community. Without the need for software installation or Unix command-line environment, these platforms are ideal for non-specialists.

In this review, we focused on web-based computational tools for enzyme engineering. We surveyed the recently developed web servers (published in 2018–2020) and tested them. We selected the ones that we considered the most outstanding and promising (Table 1) from a much larger pool of tools (Supplementary Tables S1–S6). We organized them by their focus: (i) enzyme discovery, (ii) protein solubility, (iii) enzyme activity and specificity, (iv) protein stability, (v) protein dynamics, and (vi) multipurpose. To keep the focus on enzyme design, we omitted the tools specialized in structure prediction or identification of protein–protein interactions.

Enzyme discovery

A common strategy for getting a suitable catalyst for a given substrate is to find a new natural enzyme in the genomic databases. Interestingly, there exists a vast space allowing for the discovery of novel enzymes, since the proportion of protein sequences that have not yet been biochemically characterized is enormous: only 1 in every 450 protein sequences present in the NCBI nr database [35] has a record potentially encompassing functional annotation in the manually curated UniProtKB/Swiss-Prot database [36]. Despite the availability of high throughput methods for biochemical characterization of large numbers of gene expression products [37], *in silico* approaches can conveniently reduce the process's time and costs.

Table 1

Selected web-based computational tools for enzyme engineering classified by their focus and published between 2018 and 2020. The complete list of the tools is available in the Supplementary Tables S1–S6

Web server	URL *Description*[a]	Input[b]	Output[c]	Runtime	Reference
Enzyme discovery					
HEC-net	http://hecnet.cbrlab.org *Deep learning application to predict the enzymatic activity (EC number up to its fourth level) of a protein sequence.* *Validation: Tested on a dataset of 11 353 different enzymes and 402 enzyme classes.*	- Sequence	- EC class prediction, up to the fourth level	Minutes	[3]
Bio2Rxn	http://design.rxnfinder.org/bio2rxn *Consensus prediction of the enzymatic activity (EC number up to its fourth level) of a protein sequence from six different predictive methods.* *Validation: Tested on a dataset of 3926 Escherichia coli proteins with manual annotation of EC numbers.*	- Sequence	- Reaction type - Reaction schema - EC class prediction, up to the fourth level - List of predictors agreeing with the predicted class	Hours	[4•]
GSP4PDB	https://structuralbio.utalca.cl/gsp4pdb *Search for deposited protein structures (PDB) compatible with the input graph-based structural pattern.* *Validation: Representation of a C2H2 zinc finger as a user case.*	- Graph-based structural pattern	- List of compatible PDB structures	Seconds to minutes	[5]
LIBRA-WA	http://biochimica3.bio.uniroma3.it/LIBRAWA *Identification of protein function via recognition of binding pockets.* *Validation: Tested on a set of 373 apoprotein structures.*	- PDB ID - PDB file - Ligand database - Ligand PDB ID - Search parameters	- List of PDB structures with compatible binding pocket. - Their ligands - Structural similarity score - Confidence	Minutes	[6]
EnzymeMiner	https://loschmidt.chemi.muni.cz/enzymeminer *To retrieve a list of protein sequences that potentially have the same enzymatic function than the input ones, considering their essential residue profiles.* *Validation: Experimentally validated with haloalkane dehalogenases. 658 putative hits were prioritized and the top 20 experimentally tested, leading to the discovery of several biocatalysts encompassing unique properties.*	- Sequence(s) - Essential residues	- List of putative hits annotated with multiple scores - Similarity network view	Hours	[7••]

Table 1 (*Continued*)

Web server	URL *Description*[a]	Input[b]	Output[c]	Runtime	Reference
Engineering protein solubility					
AGGRESCAN3D 2.O	http://biocomp.chem.uw.edu.pl/A3D2 *To predict the solubility of a protein from its three-dimensional structure, the effects of mutations on such structure, and the optimal substitutions for solubilization.* *Validation: The effect of dynamics in aggregation prediction tested in a set of 163 proteins. The prediction of mutation effects on solubility and stability was tested in a set of 75 globular proteins. Design of a fast-folding and aggregation-resistant green fluorescent protein.*	- PDB ID - PDB file - Mutation(s) -Non-mutable residues list - Preferences on enabling the exploration of dynamics and enhancing protein solubility.	- Aggregation profile - Per residue score - 3D view. - Effect of mutations	Minutes	[8••]
SOLart	http://babylone.ulb.ac.be/SOLART *To predict the solubility of a protein from its three-dimensional structure.* *Validation: Developed in a set of 412 proteins from Escherichia coli. Tested on 54 proteins from* Saccharomyces cerevisiae.	- PDB ID - PDB file	- Predicted solubility value - Scores of the individual features used for prediction	Seconds	[9]
AggreRATE-pred	http://www.iitm.ac.in/bioinfo/aggrerate-pred *To predict the effects of mutations on solubility.* *Validation: Assessed on experimental data consisting of 183 unique single point mutations that lead to changes in aggregation rates for 23 polypeptides and proteins.*	- Sequence - PDB ID - PDB file - Mutation(s)	- Predicted aggregation rate for each mutation	Minutes to hours	[10]
Solubility-Weighted Index	https://tisigner.com/sodope *To predict the solubility and flexibility of an input protein sequence, having the possibility of focusing only in a region of the sequence.* *Validation: Tested with the 3198 Escherichia coli proteins with annotated solubility present in the eSOL dataset.*	- Sequence	- Solubility probability - Flexibility score - Hydropathy score - Hydrophobicity and flexibility - Solubility tags suggestion	Seconds	[11•]
SoluProt	https://loschmidt.chemi.muni.cz/soluprot *To predict the solubility of a protein specified by an input sequence.* *Validation: Evaluated against a balanced independent test set derived from the NESG database consisting of 2904 proteins.*	- Sequence - List of sequences	- Solubility score	Seconds	[12•]

Table 1 (*Continued*)

Web server	URL *Description*[a]	Input[b]	Output[c]	Runtime	Reference
Engineering enzyme activity and selectivity					
FuncLib	http://FuncLib.weizmann.ac.il *To redesign an active site and create multiple-point designs. Based on conservation analysis and energy calculations.* *Validation: Applied to design a number of phosphotriesterases and acetyl-CoA synthetases with modified specificities and improved catalytic efficiencies.*	- PDB ID - PDB file - Mutable residues - Essential residues - Ligands	- List of mutants ranked by ΔΔG - PDB structures of the best designs	Hours	[13••]
CaverDock and Caver Web	https://loschmidt.chemi.muni.cz/caverweb *To calculate trajectory and interaction energy profiles of a ligand* traveling *through a protein tunnel. Available at the Caver Web interface.* *Validation: Tested with a set of enzymes with known differences in their tunnel geometries, a set of substrates with different specificities, and a set of enzymes with engineered tunnels.*	- PBD ID - PDB file - Ligand file - Ligand drawing - Ligand smiles - ZINC ligand code	- Ligand trajectory as PDBQT file - Binding energy profiles - Energy barriers	Minutes to hours	[14]
DaReUS-Loop	http://bioserv.rpbs.univ-paris-diderot.fr/services/DaReUS-Loop *For modeling or remodeling loops in homology models and finding the best loops conformation.* *Validation: Tested on dozens of examples from CASP11 and CASP12 with good or improved prediction accuracies.*	- PBD ID - PDB file - Sequence	- PDB structures of the modeled proteins - Confidence scores	Minutes to hours	[15••]
LoopGrafter	https://loschmidt.chemi.muni.cz/loopgrafter/ *For transplanting loops between two structurally related proteins, with a focus on the analysis of dynamic properties of the selected loops to transplant.* *Validation: Transplantation of a loop from* Renilla *luciferase to a reconstructed ancestor vastly improved the luminescent properties of the resulting grafted mutant.*	- PDB IDs - PDB files	- Flexibility assessment to guide the grafting process - Sequences and PDB files of the grafted proteins - Confidence scores	Minutes to hours	[16•]
nAPOLI	http://bioinfo.dcc.ufmg.br/napoli *Graph-based tool to analyze protein–ligand interactions and detect important conserved interacting residues.* *Validation: Usability demonstrated with the analysis of several crystal structures and data sets of ligand-bound proteins.*	- PDB ID(s) - PDB file(s) - Interaction cutoffs	- Interactive view of contact residues - Interaction networks - Analysis charts and tables of contacts	Minutes	[17]

Table 1 (*Continued*)

Web server	URL *Description*[a]	Input[b]	Output[c]	Runtime	Reference
Engineering protein stability					
FireProt[ASR]	https://loschmidt.chemi.muni.cz/fireprotasr *To perform ASR and infer ancestral sequences and find more stable (and promiscuous) proteins. Validation: Tested by characterization of several ancestral haloalkane dehalogenases, most of them with improved thermal stability, good activity and some with increased catalytic promiscuity.*	- Sequence - Own multiple sequence alignment - Essential residues	- Multiple sequence alignment - Phylogenetic tree - Sequence analysis and visualization	Hours	[18•]
TKSA-MC	http://tksamc.df.ibilce.unesp.br *To find hot-spots by optimizing the protein charge interactions. Calculates electrostatic free energy ΔG_{ele} of all polar/charged residues to identify destabilized ones. Validation: Applied to the cold shock protein Bs-CspB from* Bacillus subtilis *and the T4 phage lysozyme, where several predicted stabilizing mutations were confirmed experimentally.*	- PDB ID - PDB file - pH value or range - Temperature	- Electrostatic energy for every ionizable residue - ΔG_{ele} versus pH profile	Seconds	[19]
pStab	http://pbl.biotech.iitm.ac.in/pStab *To engineer protein stabilities through mutations involving charged residues. A statistical mechanical model is employed to predict the unfolding curves for the selected mutants as a function of temperature. Validation: The usage was demonstrated on ubiquitin and the haemolysin expression modulating protein (Hha), and showed some agreement with previous experimental data.*	- PDB ID - PDB file - pH - Temperature	- Pairwise electrostatic interactions - Mutational hot-spots - Stabilizing mutations and respective $\Delta\Delta G$ - Thermal unfolding curves - Local stability profiles	Minutes to hours	[20]
ProTSPoM	http://cosmos.iitkgp.ac.in/ProTSPoM *To estimate the thermodynamic stabilization $\Delta\Delta G$ upon single-point mutations using machine learning. Validation: Tested on several data sets of mutations of the tumor suppressor p53 protein with high correlations, predicted were mutations destabilizing the protein and those deleterious to its function by impairing the interaction with DNA.*	- PDB file - Mutation	- $\Delta\Delta G$ value	Seconds	[21]
Yosshi	https://biokinet.belozersky.msu.ru/yosshi *To select hot-spots for introducing disulfide bonds which naturally occur in some proteins, based on multiple-sequence alignments. Validation: Examples and benchmarking of disulfide bond predictions are given for subtilisin, myoglobin, lipases, carbonic anhydrases and xylanases.*	- PDB file - Multiple sequence alignment	- Cysteine-mutation pairs - Disulfide frequencies - PyMOL session with hot-spots - Structures of disulfide mutant	Seconds to minutes	[22•]

Table 1 (*Continued*)

Web server	URL *Description*[a]	Input[b]	Output[c]	Runtime	Reference
SSbondPre	http://liulab.csrc.ac.cn/ssbondpre *To predict disulfide bonds to enhance the protein structural stability based on machine learning and geometric restraints.* *Validation: Tested on several data sets of structures containing dozens of natural and engineered disulfide bonds. Experimentally validated with flavodoxin.*	- PDB ID - PDB file	- Cysteine-mutation pairs - Energy and entropy change	Seconds	[23]
mCSM-membrane	http://biosig.unimelb.edu.au/mcsm_membrane *To predict the stability or pathogenic effects of mutations on membrane protein and the likelihood of them being disease-associated.* *Validation: Tested with the sets containing dozens of experimentally characterized single-point mutations on several transmembrane proteins, showing accuracy levels above the alternative methods.*	- PDB ID - PDB file - Uniprot ID - Sequence - Mutation(s)	- ΔΔG value - PyMOL session with the contact maps	Seconds	[24]
DenseCPD	http://protein.org.cn/densecpd.html *To predict the probabilities of the 20 natural amino-acids for each residue in a protein structure, considering the three-dimensional density distribution of protein backbone. Uses machine learning.* *Validation: Tested with a set containing hundreds of randomly selected structures, showing accuracy higher than state-of-the-art methods.*	- PDB ID - PDB file	- Probability scores for every possible amino-acid in every position	Minutes	[25]
Engineering protein dynamics					
DynaMut2	http://biosig.unimelb.edu.au/dynamut2 *To assess changes in stability and flexibility upon mutation.* *Validation: Tested with an independent dataset consisting of 611 single point mutations derived from ProTherm database, a test set of 276 mutations with low sequence identity to proteins in the original ProTherm dataset, and an independent test set comprising 173 variants in six proteins with experimental melting temperatures changes.*	- PDB ID - PDB file - Mutation(s)	- ΔΔG value - 3D view (wild type or mutant) with predicted interactions of the mutated residue(s) and B-factor and hydrophobicity mapping - B-factor profile	Seconds to minutes	[26•]
CABS-flex 2.0	http://biocomp.chem.uw.edu.pl/CABSflex2 *To evaluate the flexibility of the input protein structure.* *Validation: The method was compared to the classical, all-atom molecular dynamics on 22 different proteins. The calculated relative fluctuations were shown to be well correlated to 140 different NMR ensembles.*	- PDB ID - PDB file - Execution parameters	- Ensemble of structure files for all normal modes. - Contact (cross-correlations) map - Fluctuations plot	Minutes to hours	[27]

Table 1 (*Continued*)

Web server	URL *Description*[a]	Input[b]	Output[c]	Runtime	Reference
ProSNEx	http://prosnex-tool.com *To evaluate the flexibility of the input protein structure.* *Validation: The effectiveness of the approach is illustrated on the use case of TEM-1 β-lactamase.*	- PDB ID - PDB file - Execution parameters	- 3D view mapping calculated properties on the structure - Network analysis - Cross-correlation maps	Minutes to hours	[28•]
AlloSigMA 2	http://allosigma.bii.a-star.edu.sg *To evaluate the allosteric effects of ligand binding or mutations.* *Validation: Benchmarked on a dataset consisting of a total of 52 proteins with 60 allosteric sites.*	- PDB ID - PDB file - Mutation(s) - Ligand-binding site	- Per residue ΔΔG value - Allostery modulation graph for the effects of ligand binding or mutation.	Minutes	[29]
LARMD	http://chemyang.ccnu.edu.cn/ccb/server/LARMD *To perform short-timed fully atomistic conventional (Int_mod) and steered (str_mod) molecular dynamic simulations, and normal mode analysis (nor_mod) for the study of ligand binding and unbinding.* *Validation: The usefulness illustrated with a selective mechanism of the β-type estrogen receptor, which plays a vital role in the treatment of inflammatory diseases and many types of cancers.*	- PDB ID - PDB file - Ligand	- Ensemble of structure files representing the trajectory. - PCA analysis, conformation clusters and dynamic residue cross-correlations (int_mod) -Hydrogen bond analysis and energy decomposition if protein–ligand is submitted (int_mod) - Tunnel and transport energy profile (str_mod) - Cross-correlations and residues fluctuations map (nor_mod)	Int_mod: minutes, Str_mod: hours	[30•]
Multipurpose					
Caver Web	https://loschmidt.chemi.muni.cz/caverweb *To calculate tunnels in proteins with buried binding sites and analyze the ligand transport through those tunnels.* *Validation: Applied on several haloalkane dehalogenases with well-known tunnel properties, the binding of paracetamol to cytochrome P450 3A4, and the virtual screening of leukotriene A4 hydrolase/aminopeptidase inhibitors. Utility can be inferred by the many articles that used CAVER 3.0 as a key instrument for enzyme design.*	- PBD ID - PDB file - Ligand file - Ligand drawing - SMILES - ZINC codes	- Enzyme cavities - Enzyme tunnels - Tunnel profiles - PyMOL session with tunnels - Tunnel residues - Ligand energy profiles - Energy barriers - ligand trajectory	Pockets: seconds, tunnels: seconds, ligand transport: minutes to hours	[31••]
HotSpot Wizard 3.0	http://loschmidt.chemi.muni.cz/hotspotwizard *For automated identification of hot-spots in semi-rational protein design to give improved protein stability, catalytic activity, substrate specificity and enantioselectivity. It can estimate the stability effect of specified mutations and design smart libraries.* *Validation: Tested by predicting the stabilization effects of thousands of experimentally characterized single- and multiple-point mutations, for correctly identifying a previously known mutation as the most stabilizing substitution, and for the automated design of tunnel-engineered variants with single- and multiple-point mutations.*	- PDB ID - PDB file - Sequence	- Enzyme pockets - Enzyme tunnels - Multiple sequence alignment - Homology models - Correlated residues - Amino-acid frequency - Mutational effect on function - Flexibility (B-factors) - Mutability scores -Sequence consensus - ΔΔG of designed mutants - Library design for saturation mutagenesis	Analysis: hours, mutations design: minutes to hours, library design: seconds	[32•]

Table 1 (*Continued*)

Web server	URL *Description*[a]	Input[b]	Output[c]	Runtime	Reference
ProteinsPlus	https://proteins.plus *To search, validate, analyze and predict multiple properties and features of proteins, their binding sites and interactions with ligands.* *Validation: Demonstrated with test examples of the human deoxy hemoglobin and matrix metalloprotease-13, and several tutorials available at the server.*	- PDB ID - PDB file - Ligand file - Keyword search	- Hydrogen prediction - Protein pockets and binding sites - 2D interaction diagrams - Ensemble compilation from PDB - Protein–protein interaction analysis - Metal coordination prediction - Protein–ligand affinity or activity - Placement of water molecules in the active site - Structure quality assessment	Seconds to minutes	[33•]
pPerturb	http://pbl.biotech.iitm.ac.in/pPerturb *To quantify the strength of an interaction network by employing perturbations (alanine mutations); can predict the extent of destabilization of proteins arising from side-chain truncations.* *Validation: Usage examples for the human Neurotensin Receptor 1, acyl-CoA-binding protein (ACBP), synaptic protein 95, phosphofructokinase, and ubiquitin.*	- PDB ID - PDB file - Target residue	- Perturbation profiles - Contact network plots - Allosteric hot-spots - Thermal unfolding curves	Minutes	[34]

[a] Brief description of the tools and their experimental validation, when available, or usage examples.
[b] Some of the listed items are mandatory and some are optional.
[c] $\Delta\Delta G$ is the stabilization energy, and corresponds to the change in free energy upon each mutation from the wild-type or template. All the web servers listed were tested and were fully functional at the final stage of this manuscript.

The task of discovering new enzymes can be tackled in different manners. A straightforward strategy is to predict the enzymatic activity of a protein from its sequence. HEC-Net [3] is a deep learning tool that exploits strategies based on sequence pattern recognition, sequence similarity, and amino-acid biochemical properties to achieve prediction accuracy over 90% on the fourth level of the Enzyme Commission (EC) classification. Also exploiting deep learning, Bio2Rxn [4•] produces a consensus prediction based on six individual predictors. One of them is based on convolutional neural networks that are trained exclusively on EC-number annotated protein sequences. The other five are more traditional predictors based on sequence similarity, identification of sequence patterns, and amino-acid biochemical properties. Bio2Rxn retains high precision values (over 90%) while increasing recall (close to 60%) compared to similar tools.

A complementary strategy consists of identifying protein–ligand structural motifs from the protein structures in RCSB Protein Data Bank (PDB) [38]. This approach relies on the rationale that such binding interfaces are substrate-specific, which in turn is an essential fingerprint of the catalytic process. GSP4PDB [5] allows the user to design and define the so-called Graph-based Structural Patterns as the protein–ligand interface representations and then query for such patterns in the PDB, thus returning proteins that could potentially accommodate the ligand. LIBRA-WA [6] is a web-based application that exploits network theory to identify binding pockets in an input protein. Such identification is done upon comparison with two precompiled databases, ligand-binding sites, and the Catalytic Site Atlas [39].

A third strategy consists of finding the existing protein sequences that could carry on an enzymatic function. While the first approach relied on precisely predicting the enzymatic function of an input sequence, the challenge here is to comprehensively identify the maximum number of protein sequences able to perform a given catalytic function. EnzymeMiner [7••] accepts several proteins with known enzymatic function as input, infers their essential or catalytic residues, and exploits different tools for the assessment of sequence similarity to identify such potential catalysts (Figure 1). In contrast with the second strategy tools, EnzymeMiner does not rely on knowledge of the 3D structure of proteins. The tool is fully automated, ranks sequences by their predicted solubility, and provides annotations on source organism, extremophilicity, structure availability, and so on, to guide the selection process.

Engineering protein solubility and aggregation

A recurring problem with producing engineered proteins is that they may suffer from diminished solubility or increased aggregation. Several approaches relying on sequence and structure properties provide solutions for solubility prediction and optimization [40,41].

Among the methods requiring the input of 3D structure, Aggrescan3D 2.0 [8••] is a well-established aggregation predictor that projects a pre-calculated intrinsic aggregation propensity scale to the query protein structure. Thus, the aggregation propensity values used to produce the final prediction are modulated by the specific structural context of the evaluated region or patch. The newest version improves the predictions by considering protein flexibility and stability and providing optimized solubility suggestions.

SOLart [9] relies on structure-derived statistical potentials to infer the query protein solubility. Differences in Gibbs free energy inferred from such statistical potentials – especially those considering backbone torsion angles, solvent accessibility and inter-residue distances – allow for accurate predictions of solubility when compared with experimental values, achieving a Pearson's correlation coefficient of 0.67 and 0.51 on independent validation set and modelled proteins, respectively.

AggreRATE-pred [10] integrates amino-acid physicochemical and structural-based properties, and mutational and contact propensities in a multiple regression model to predict the effect of mutations on the aggregation rates. The chosen model to be applied depends on the protein length and the secondary structure type on where the mutation(s) occur. This strategy achieves a correlation between experimental and predicted values of up to 0.82 and performs well on modeled proteins. Interestingly, this approach does not rely on any structural information for short peptides (< 40 amino-acids).

When the 3D structure of the protein to engineer is not available or obtaining a model becomes challenging, solubility can also be predicted from the protein sequence. Solubility-Weighted Index [11•] offers a precalculated compendium of per-residue flexibility propensities that were refined and optimized in a set of 12,216 target proteins from 196 different species that were expressed In *Escherichia coli* using either a C-terminal or N-terminal poly-histidine fusion tag. The strategy derives from the observation that, over almost 10 000 different studied protein properties, flexibility was the best predictor for solubility.

SoluProt [12•] is based on gradient boosting regression and provides solubility prediction from the protein sequence. The machine learning model has been developed using a manually curated TargetTrack database. Considering the amino-acid singlet and dimer content of the poly-peptidic chain, their physicochemical properties, membrane propensity, and similarity to *E. coli* 3D

Figure 1

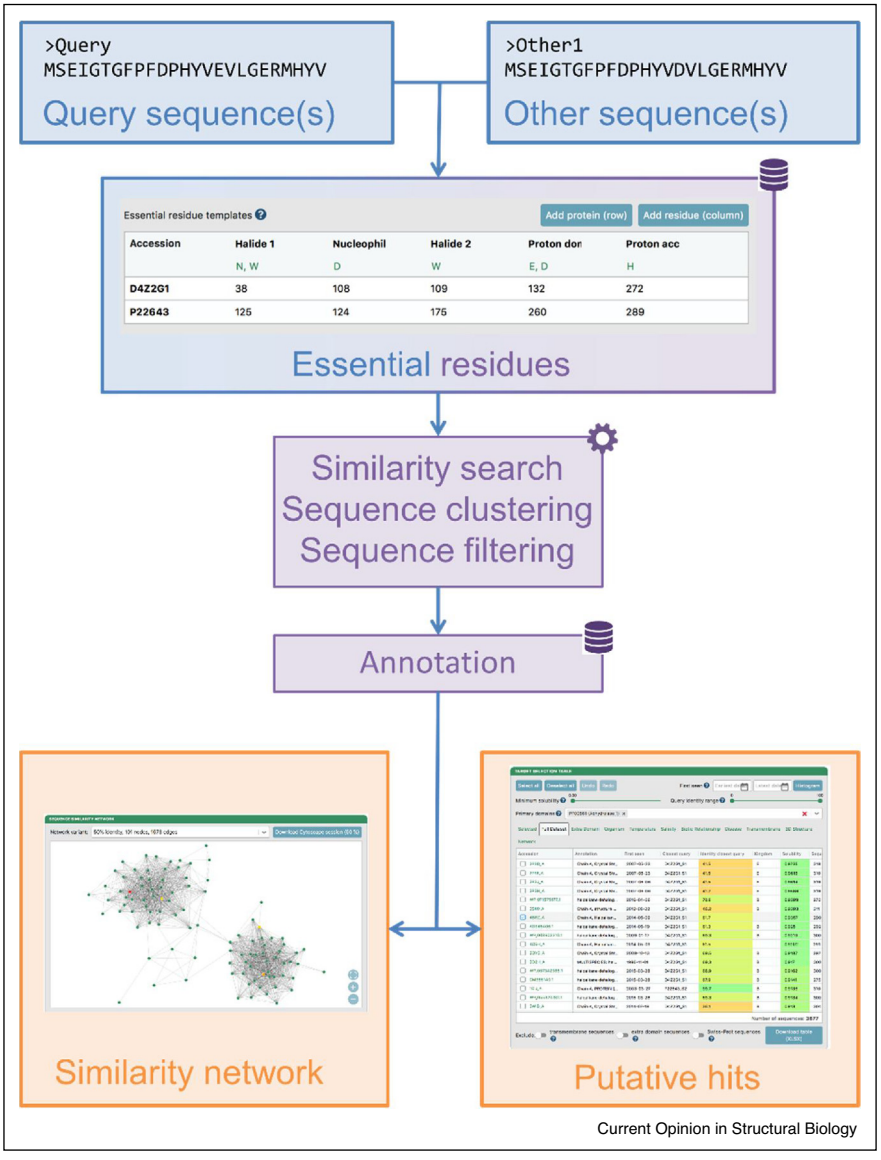

Illustration of the EnzymeMiner workflow [7••]. The web server accepts several sequences with the desired function. The user can also input 'other sequences' performing the desired function to help on the sequence filtering step. The server can retrieve catalytic residues from the Catalytic Site Atlas, or the user can define them (allowing for degenerated positions). The query proteins are used to search for homologs, and the obtained hits are subsequently clustered and filtered, ensuring the presence of the defined essential residues. Multiple annotations are retrieved to enrich the information of the filtered list of hits. The final results are presented in two interactively integrated views: (i) Putative Hits allows for prioritization according to any of the retrieved annotations, and (ii) the Similarity Network view presents the sequences clustered according to their sequence similarity.

proteome, this approach achieves an AUC of 0.60 on a newly compiled independent set. SoluProt is integrated in EnzymeMiner [7••], providing an easy way to filter out unlikely soluble proteins in the process of novel enzyme discovery.

Engineering enzyme activity and specificity

Enzyme activity and selectivity are the key features typically targeted in enzyme engineering. Although enzyme activity and selectivity are very different properties, they can often be improved using similar computational approaches. Engineering the activity towards a substrate of interest is also likely to enhance the selectivity towards this substrate. The most common strategy consists of introducing mutations in the active site and optimizing it towards the targeted substrate. Other approaches have also proven successful, namely the engineering of access tunnels, modification of the dynamic properties, editing recognition elements such as loops, or targeting allosteric sites.

Important computational tools for engineering enzyme function – among which is the gold-standard Rosetta toolbox [42] – have been reviewed [43,44]. Rosetta-based web tool FuncLib [13••] was specially designed to add multiple-point mutations to the binding site. After performing evolutionary analysis and energy calculations, single-point mutations are combined and ranked by the predicted stabilization free energies (ΔΔG). The FuncLib workflow ensures that no deleterious mutations are introduced, and it can account for potential epistatic effects resulting from combining multiple mutations.

CaverDock [14], integrated into the Caver Web [31••] (Section 'Engineering multiple properties'), can be used for engineering enzyme activity and selectivity. This tool was designed to predict the trajectory and energy profile of (un)binding of a ligand travelling through the enzyme access tunnels using a constrained molecular docking algorithm. The user can run calculations for different ligands or multiple enzyme variants and assess which combinations provide the best energy profiles. This is especially useful when the limiting steps in the catalysis involve the substrate binding or the product release.

Enzyme specificity can also be modified by engineering loops, which represent the flexible elements modulating substrate recognition and binding specificity. DaReUS-Loop [15••] models loops in homology models, and it can search the databases for new loop conformations suitable to be introduced in the target structure. It can help users find new enzyme variants with diverse substrate specificities. LoopGrafter [16•] aims to transplant loops between two structurally related proteins, while evaluating feasibility of potential solutions. The protocol focuses on analyzing the geometrical similarity and the dynamic properties of the transplanted loops, and provides graphical guidance on the process. All possible insertion points for the selected loops that generate different sequences are evaluated, providing the user with means to rationally engineer ligand-recongnition elements and protein dynamics (Section 'Engineering protein dynamics').

nAPOLI [17] automatically identifies conserved protein–ligand interactions across a large data set, such as a list of PDB structures or any protein within a specified range of sequence identity. It compiles the type of interactions and networks formed to find hot-spots within the binding sites or suggest mutations that can produce more favorable interactions with a specific substrate.

Engineering protein stability

Enzyme stability refers to the range of temperature, co-solvents, pH, and other general conditions in which enzymes can resist and remain active. It is desirable for many biotechnological purposes that the enzymes survive longer time or harsher conditions beyond what the native variants normally could. One can push those boundaries by engineering their stability using: (i) energy calculations, (ii) phylogenetic analysis, (iii) machine learning, and (iv) a combination of the previous ones. These strategies [45–48] and software tools [41] have been extensively reviewed.

Ancestral sequence reconstruction (ASR) is a strategy that is becoming increasingly used for protein stabilization. FireProt^{ASR} [18•] is the first fully automated platform for inferring the ancestral sequences by phylogenetic analysis. Based on a single protein sequence, the tool builds a data set of homology sequences and performs a multiple sequence alignment to construct a phylogenetic tree and reconstruct the ancestral nodes. The method can be used not only to improve thermostability, but also to expand the catalytic promiscuity and increase expressibility of enzymes.

Electrostatic interactions are crucial to protein folding and integrity. They also rule the effects of pH and ion concentration on protein stability. However, they are often underestimated or poorly predicted during enzyme engineering. TKSA-MC [19] and pStab [20] tools tackle this issue by assessing unfavorable electrostatic interactions and identifying charged hot-spot residues for mutagenesis.

A very different approach to protein stabilization is the introduction of disulfide bonds. Yosshi [22•] and SSBondPre [23] are recent tools devoted to this strategy, the former using evolutionary analysis and the latter using machine learning. Most of the stability prediction methods have been developed for globular soluble proteins. mCSM-membrane [24] can predict the stability changes or the pathogenicity associated with mutations in membrane proteins.

Engineering protein dynamics

Proteins exist in dynamic, metastable conformational states, transitioning through an ensemble of possible local conformations. The motions resulting from such transitions can fundamentally influence the catalytic activity of an enzyme [49,50]. Thus, assessing and engineering enzyme dynamics may be crucial for achieving the desired activity output. It also has an impact on predicting protein solubility and stability.

DynaMut2 [26•] combines Normal Mode Analysis methods and graph-based signatures to investigate the effects of single-point and multiple-point mutations on protein stability and dynamics. The server reports B-factors that characterize the predicted flexibility of the mutants and the changes in stability. Moreover, the server offers the possibility to independently run coarse-grained predictions on a structure using five different force fields.

CABS [51] is a coarse-grained force field accounting for side-chain contacts, main-chain hydrogen bond networks, and local geometric preferences. It was validated against molecular dynamics and nuclear magnetic resonance ensembles and is part of AGGRESCAN 3D [8••]. Freshly re-implemented in a web server CABS-flex 2.0 [27], it allows for evaluating larger proteins with up to 2000 residues, for imposing user-defined distance restraints, and offers an improved graphical output.

ProSNEx [28•] models inter-residue interaction networks from the input 3D coordinates of the protein to be studied. Such contacts are weighted according to dynamical cross-correlation maps, either obtained from elastic network models or other normal mode applications, the graph theory-based spectral clustering of side-chains, or molecular dynamic simulations derived energies. These dynamics studies are enriched with subsequent network and sequence conservation analysis, and the results are presented in an easy-to-interpret graphic-intensive interface.

AlloSigMA 2 [29] studies allostery and is based on implementing a structure-based statistical mechanical model. The server allows for evaluating the allosteric free energy resulting from the perturbation of any residue in the input structure. It allows for testing the allosteric effects of introducing mutations and the impact of introducing a ligand into the studied system. An intuitive graphical user interface provides a rapid interpretation of the protein regions that changed their dynamics.

LARMD [30•] automates the execution of fully atomistic molecular dynamics simulations up to 4 ns long. Untrained users can opt for the suggested easy-to-set-up predefined conditions, and more versed ones can fine-tune the execution parameters to their needs. The application is focused on deciphering the structural and dynamical effects of ligand binding, and to this end, implements tunnel discovery tools such as CAVER 3.0 [52]. Furthermore, it offers a wide range of analyses on the obtained trajectories: (i) structural variability and fluctuation analyses, (ii) normal mode analysis, and (iii) trajectory clustering. The server provides a wide range of graphics and charts to ease the interpretation of the results.

Engineering multiple properties

Some protein engineering web-tools integrate multiple tasks in robust workflows. Caver Web [31••] can be used to identify molecular tunnels and channels in proteins with buried cavities and predict the transport of ligands through these tunnels (Figure 2). The workflow starts

Figure 2

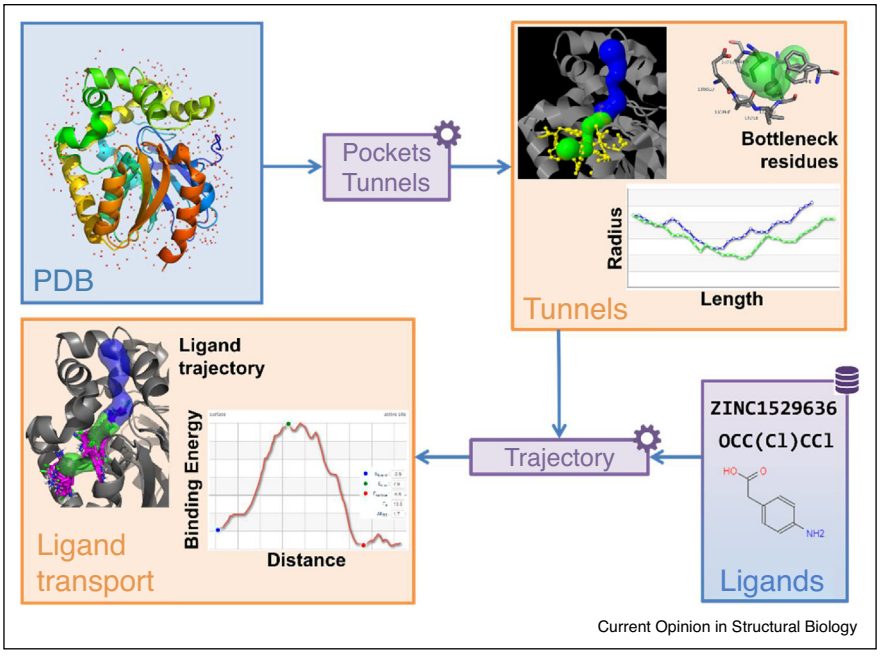

Illustration of the Caver Web workflow [31••]. The user enters a PDB file or PDB code. The pockets in the 3D structure are calculated and one of them is used as a starting point to calculate the tunnels to the surface. The identified tunnels can be analyzed for their properties, bottleneck residues and tunnel-lining residues. The user can enter one or multiple ligands as files, drawing, SMILES or ZINC codes, and calculate their trajectories through the selected tunnels. The user can analyze the binding energy profiles of the ligand, determine energy minima, maxima and energy barriers. The ligand trajectory and the list of bottleneck residues forming the energy barriers can be downloaded.

Figure 3

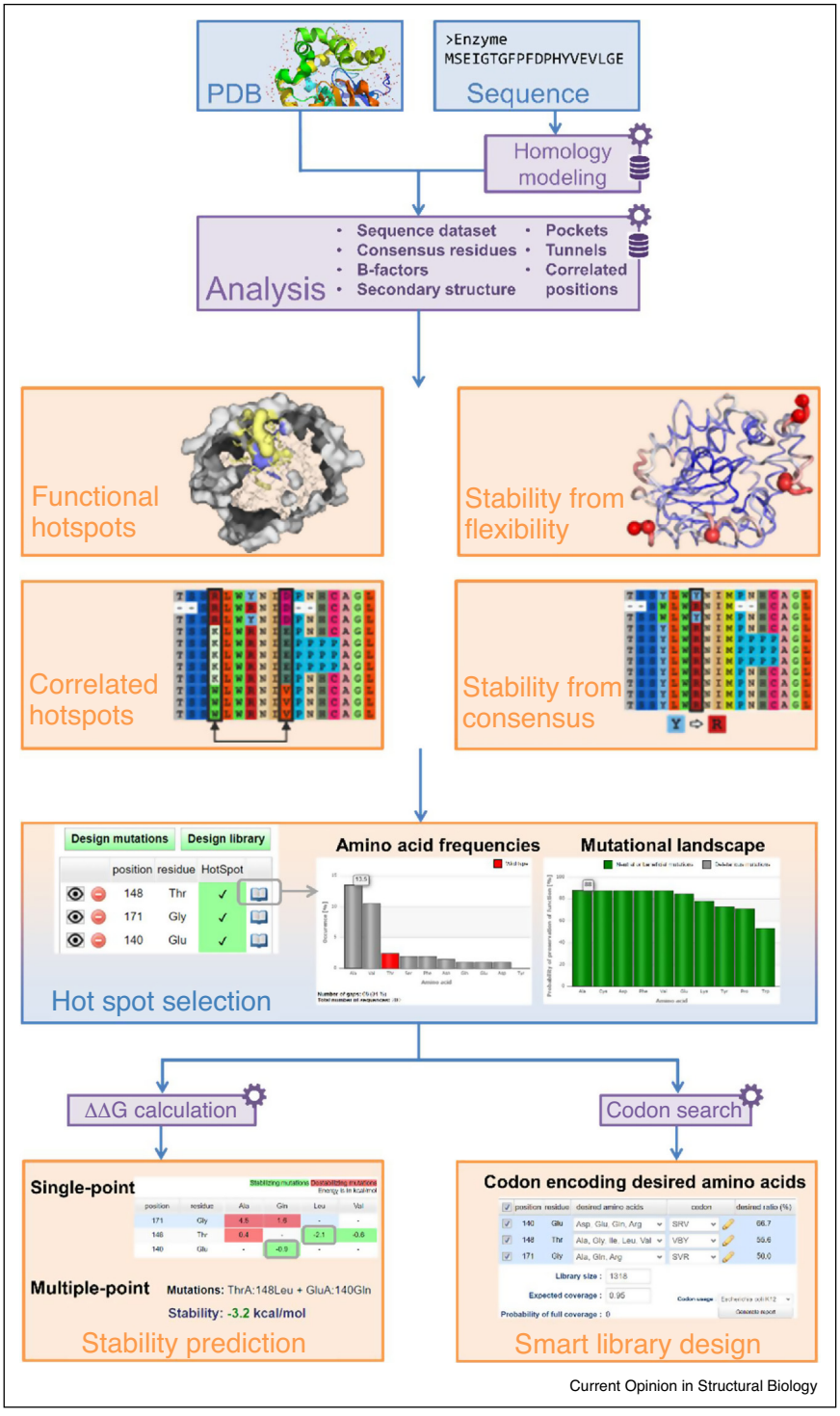

Illustration of the HotSpot Wizard 3 workflow [32•]. The user enters a PDB structure, or a sequence that will be used to predict the structure by homology modeling. A sequence of different calculations are performed, leading to four types of hot-spot predictions: (i) functional hot-spots (non-essential residues located on functional pockets or tunnels, ranked by mutability), (ii) correlated hot-spots (co-evolving pairs of residues, obtained from consensus and correlation analysis), (iii) stability from flexibility (hot-spots with higher B-factors), and (iv) stability from consensus (hot-spots recommended to be mutated to amino-acids with higher frequency in the multiple sequence alignment). The user can select the hot-spots for mutagenesis based on the integrated overview of the suggested positions, such as mutability, secondary structure, amino-acid frequency and mutational landscape. The user can predict the stabilization (ΔΔG) from all the selected single-point mutations on the selected hot-spots and combine them into multiple-point mutations. The user can also calculate the optimal DNA codon content to build smart libraries for screening the selected positions with the desired set of amino-acids.

with identifying the relevant pockets and computing the tunnels from the selected pocket to the surface using CAVER 3.0 [52]. The user then selects the tunnels and ligands to analyze the transport using CaverDock (Section 'Engineering enzyme activity and specificity'). This integrated analysis allows identifying hot-spots on the enzyme tunnels that can remove the barriers to the transport of the target substrates or products or increase their specificity, thus improving the enzymatic function.

HotSpot Wizard 3 [32•] is a tool for the identification of mutagenesis hot-spots for improving stability, activity, and specificity, following a multi-stage automatic workflow (Figure 3). The tool sequentially calculates several parameters to identify: (i) functional hot-spots located in the active site pocket and/or access tunnels, (ii) stability hot-spots corresponding to flexible residues, (iii) stability hot-spots based on back-to-consensus, and (iv) correlated hot-spots corresponding to co-evolving residue pairs. Recent updates have made possible the calculation of homology models from the protein sequence. The user can build smart libraries based on the amino-acid frequencies, predict the stabilization energy of selected mutations, and even combine the interesting ones into multiple-point mutations.

ProteinsPlus [33•] is a unified platform integrating multiple protein investigation tasks, namely database exploration, structural quality assessment, conformational analysis, binding site analysis, 2D-interaction diagrams, pocket detection, and so on. Although it is not devoted to enzyme engineering *per se*, it can provide comprehensive structural knowledge.

pPerturb [34] aims primarily at assessing the importance of different residues to the stability by analyzing the effects of alanine mutations on the global number of contacts in the structure. The workflow is divided into perturbation profiles calculation (ΔQ), interaction networks, and the change in thermodynamic stability from truncating side chains. Overall, the tool can facilitate identifying residues that determine local stability and potential allosteric signal transduction pathways.

Conclusions and perspectives

Here we reviewed the recently published web-based tools specialized in different aspects of enzyme engineering, which can be valuable resources to experimental scientists. The advantages of web-based tools are their immediate use without tedious installations, optimal settings already selected by the developers, regular updates and maintenance, and shared computational resources. We observed a boom of new methods and approaches, especially the rise of predictors based on machine learning, for which the quality of the experimental data used for training is of paramount importance. However, this is not always guaranteed by the available databases, which would highly benefit from stronger efforts of the community to supply high-quality, findable, annotated and curated data. These data will also provide essential input for machine learning as well as critical comparisons of newly developed tools. Modern high-throughput experimental technologies like fluorescent activated cell sorting, microfluidics, cell-free expression, and deep mutational scanning will enable collecting large and highly consistent data sets.

We observed many tools devoted to enzyme discovery, although mainly focused on predicting the potential enzymatic activity of a protein sequence, but not for retrieving potential catalysts from a collection of orphan proteins. We also see a shift in the strategies for engineering activity and specificity, as many recent tools focus on non-active site elements, for example, loops, tunnels, highly flexible and allosteric regions. In general, tools for engineering catalytic activity, selectivity and protein solubility are insufficiently developed, and significant improvements are needed to provide more accurate and practically useful predictions. With the constant increase of computational power, which allows a more robust assessment of structural ensembles, we expect protein dynamics to become a more integral part of the next-generation tools. We predict the same should happen with the design of catalytic activity using high-level methods, that is, quantum mechanics or hybrid quantum mechanics/molecular dynamics, to be made accessible via web servers. We have witnessed a game-changing situation with the development of GPU cards and their use for computationally demanding tasks. We envisage another breakthrough with the gradual maturation of quantum computing. Once fully operational, such technology will boost the current computational capacity by several orders of magnitude, allowing the routine use of high-level theory calculations and extensive combinatorics.

Conflict of interest statement

Nothing declared.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.sbi.2021.01.010.

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