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Annotation of Proteins of Unknown Function: Initial Enzyme Results

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Abstract

Working with a combination of ProMOL (a plugin for PyMOL that searches a library of enzymatic motifs for local structural homologs), BLAST and Pfam (servers that identify global sequence homologs), and Dali (a server that identifies global structural homologs), we have begun the process of assigning functional annotations to the approximately 3,500 structures in the Protein Data Bank that are currently classified as having “unknown function”. Using a limited template library of 388 motifs, over 500 promising *in silico* matches have been identified by ProMOL, among which 65 exceptionally good matches have been identified. The characteristics of the exceptionally good matches are discussed.

Keywords

bioinformatics; catalytic motif; enzyme; ProMOL; protein function; PyMOL; structural biology

Introduction

Elucidating the functions of proteins is a core component of biochemistry, structural biology, and bioinformatics. Scientists in these disciplines seek to understand the relationship among protein sequence, structure, and function. A recent search identified approximately 3,500 structures of “unknown function” in the Protein Data Bank (PDB) [1, 2]. Software tools have been developed to relate sequence, structure and function. Several programs exist that can propose functional annotations for a specific target of interest. Sequence databases can be searched with tools such as BLAST [3] and HMMER [4] to identify sequence homologs. Databases, repositories, and servers such as UniProt [5], Pfam [6, 7], the Structural Biology Knowledgebase [8], Dali [9], and MarkUs [10] collect and display information from various sources that may be used to identify structural homologs and/or give functional insight.

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Compliance with Ethical Standards

None of the authors have reported any conflicts of interest in the completion of the research described in this manuscript.

We have developed the ProMOL [11] plugin for PyMOL [12], a tool used to explore the catalytic site structural homologies between proteins of known function and those for which functions are not yet known. ProMOL uses template-based alignment of these structures with a current library of 388 active site motifs as reported in the Catalytic Site Atlas [13]. We have applied this approach to examine structures in the Protein Data Bank which are listed as having "unknown function". Although catalytic site structural homology alone is not sufficient to define the function of a protein, it provides one mechanism which, when combined with other structural and sequence motifs, can suggest candidates for experimental verification. We also applied three well-established methods for protein function assignment based on sequence (BLAST, Pfam) and global structure alignments (Dali) to gain more insight into the functions proposed by ProMOL. ProMOL is being developed collaboratively and distributed freely as open source software (http://sourceforge.net/projects/sbevsl/ or http://www.promol.org).

The aim of this study is to analyze all uncharacterized PDB entries using ProMOL to identify potential enzymatic functions for some of these structures. Targets showing high similarity to known catalytic sites using ProMOL were then analyzed using additional *in silico* methods (BLAST, Pfam, and Dali) in an attempt to identify potential enzymatic functions. Of the 3,437 PDB entries of "unknown function" that have been processed with the current ProMOL motifs, over 500 entries yielded high-probability matches (≥3-residue alignments with RMSD <10 Å for non-hydrogen atoms). There were 65 exceptional matches (3-residue alignments with RMSD ≤2.5 A or 4- and 5-residue alignments with RMSD ≤4.0 A) against the current motif library.

Methods

The search of the PDB for proteins of "unknown function" was performed with an advanced text search for entries containing "unknown function". An additional search using the above criteria plus a deposition date between 1/1/2000 - 7/7/2014 was also performed, indicating that 96.4% of these structures have been generated since the advent of the Protein Structure Initiative (PSI) [14].

We have created a library of 388 motif templates containing well-defined enzyme active sites, as found in the Catalytic Site Atlas [13]. The motif templates span all six top-level EC number groups to at least the third level (e.g., 3.1.21.*) wherever structures in those groups are available. Structures were selected based on the availability of literature definitions of active sites in the Catalytic Site Atlas [15]. Two sets of motif templates make up the current library: roughly half were generated manually with the Motif Maker tool in ProMOL [11], which can be used to create motif templates based on residue name, residue number and chain identifier for the residues in the active site of an enzyme structure found in the PDB. The remaining motif templates were generated automatically with a script that tests the motif template against itself, homologs and random PDB structures to provide sensitivity and specificity data for each new motif template [manuscript in preparation].

These structures were screened according to the protocol illustrated in Figure 1. The first step was to run all of the uncharacterized proteins through ProMOL to compare the identity and geometry of the amino acid residues in these structures with the motif library. Results which met the following criteria were subjected to further analysis: (1) 3-residue alignments with RMSD ≤ 2.5 Å (for non-hydrogen atoms) or (2) RMSD ≤ 4.0 Å (for non-hydrogen atoms) for ≥ 4-residue alignments (RMSD values were calculated in three ways: C_α used only the alpha carbons; C_α and C_β used the alpha and beta carbons; and All used all the non-hydrogen atoms in the residues). In an attempt to narrow our search results to structures with reliable functional annotations, the sequences of hits from our initial search were used to search the PDB with BLAST. The protein sequences were also evaluated with Pfam to gain additional functional insight. Finally a global structural alignment was conducted with Dali. The results from these four approaches (ProMOL, BLAST, Pfam, and Dali) were then compared and evaluated to predict the most likely function for the proteins.

Ligand binding was conducted with AutoDock Vina [16] according to the instructions on their web site (http://autodock.scripps.edu/). Once a protein had an assigned EC number, ligands that were bound to PDB entries under that same EC number were selected for *in silico* binding studies. Ligands and protein structures were converted to *.pdbqt files using AutoDockTools. The grid box for the enzyme active site was defined based on the location of catalytic residues predicted by ProMOL. AutoDock Vina was executed from the command prompt and the results were then visualized in PyMOL. Each docking experiment was repeated four times and the average free energy values are reported in Table 2.

Once a putative function is established, literature searches are conducted for enzymes from these families so that suitable assay conditions and substrates may be found. Plasmids that can express these proteins must be obtained from sources such as DNASU [17–19]. Substrates are then ordered from commercial suppliers such as Sigma-Aldrich. The protein can be expressed in *E. coli* and isolated using established methods of protein production and purification. The predicted molecular weight is confirmed using SDS-PAGE. Enzyme activity assays are then conducted using appropriate substrates.

Results and Discussion

As of 31 October 2014 there were 3,646 structures of “unknown function” among the more than 100,000 structures in the PDB. The initial search in May 2013 yielded 3,437 structures, which are described below; any newly deposited structures which are classified as “unknown function” will be characterized with new versions of ProMOL containing more motif templates. The 3,437 proteins were analyzed with ProMOL, BLAST, Pfam, and Dali and over 500 promising hits (15%) were identified. It should be noted that about 58% of current entries in the PDB have Enzyme Commission (EC) numbers; if the same probability exists for structures of unknown function, then roughly 2,000 of them would be expected to have enzyme function. One reason for this discrepancy is the limited number of motif templates that are currently available. As mentioned above, our motif template library is being expanded and will be used to further characterize the remaining structures of unknown function. It is also possible that a significant number of these proteins have enzymatic activity that has not been previously characterized and for which no homologous structures exist.

The set of 500 promising hits (≥3-residue alignment with RMSD <10.0Å) was further distilled down to 65 exceptional hits (3-residue alignment with RMSD <2.5A or ≥4-residue alignment with RMSD <4.0Å). These results are presented below in a series of tables and in the supplemental materials. Table 1 highlights results with agreement of functional annotations among ProMOL, BLAST, Pfam, and Dali. Table 2 summarizes the cases in which ProMOL provided promising alignments compared to inconclusive results provided by BLAST, Pfam and Dali. The results in Table 3 are cases where BLAST, Pfam and Dali suggested similar potential functions, while ProMOL suggested a clearly different function. Table 4 contains inconsistent results, i.e., the predicted functions from the four programs were not in agreement. Table 1S provides the full list of results for each of these comparisons,

Uniform Function Assignment

Of the 65 structures, there were 13 cases in which ProMOL, BLAST, Pfam, and Dali all assigned similar probable functions, agreeing at least on the first digit in the EC number; in many cases agreement extended to additional digits. For example, in the case of 2AQW [20], all four programs assigned the structure to EC number 4.1.1.23. This level of agreement among the four bioinformatics programs indicates that 2AQW is a strong candidate for *in vitro* characterization.

High quality five-residue matches between a query structure and a motif template are rare, primarily because most enzyme motifs in the ProMOL library do not contain five residues within the active site. The alignments of 3L1W (query) [21] with 1AKO (motif template) [22] and 2O14 (query) [23] with 1BWR (motif template) [24] are uniquely interesting because both structures exhibit five-residue alignments with their respective motif templates and had RMSDs < 1.0 Å.

Figure 2A depicts the five-residue alignment of 3L1W (query) with the 1AKO motif template (hydrolase, EC 3.1.11.2). The RMSD for all non-hydrogen atoms / C_α / C_α + C_β was 0.25 / 0.16 / 0.17 Å, respectively. Pfam, BLAST, and Dali all indicated that 3L1W was most likely an endonuclease, exonuclease, or phosphatase with a likely EC number either 2 or 3.1 (Tables 1 and 1S). The matching motif template, 1AKO, has been identified as an exonuclease III [22], which is involved in the removal of abasic sites in *E. coli* DNA. Finally, a global structural alignment using Dali (Figure 2B) reveals a fairly high level of full backbone structural conservation between 3L1W and 1AKO: *Z*-score 20.8, RMSD 2.9 Å (covering 213 residues of the 268 total residues in 1AKO with only 18% sequence identity).

A second five-residue alignment was also found between 2O14 (query molecule) and 1BWR (motif template), an acetylhydrolase. The ProMOL alignment consisted of residues SER171, GLY209, ASN241, ASP339, and HIS342 (for the query, 2O14) and residues SER47, GLY74, ASN104, ASP192, and HIS195 (for the template, 1BWR). The RMSD values for all non-hydrogen atoms / C_α / C_α + C_β were 0.58 / 0.51 / 0.71 Å, respectively. BLAST characterizes 2O14 as an esterase (EC 3.1.1), while Pfam characterizes it as a GDSL-like lipase/acylhydrolase (EC 3.1). Dali characterizes 2O14 as a rhamnogalacturonan acetylesterase, or putative lipase (EC 3.1). These results represent general agreement to the first two EC numbers, as hydrolases that act on ester linkages; *in vitro* characterization is needed to resolve the subtle differences.

Consistency between ProMOL, BLAST, Pfam, and Dali for these 13 targets makes the structures in Table 1 strong candidates for *in vitro* characterization. Some of these structures are currently being analyzed in our labs.

Unique ProMOL Assignments

Of the 65 structures of unknown function that generated significant “hits” when screened through ProMOL, there were fifteen cases in which BLAST, Pfam, and Dali all returned inconclusive results. These programs either assigned the structures to DUF (domain of unknown function), compared them to other uncharacterized or “hypothetical” proteins, had inconclusive matches, or simply returned no results. The results obtained with ProMOL are listed in Table 2. The first column is the structure of unknown function, while the second column provides the PDB ID for the motif template that provided the alignment results and the suggested function, followed by the EC number of the motif template, RMSD for all non-hydrogen atoms, and the proposed function, as determine by ProMOL. The last three columns reflect ligand binding to the query proteins.

To explore these unique ProMOL alignments more deeply, we selected ligands that were bound to PDB entries under the same EC number. Autodock Vina [16] was used to estimate the free energy and dissociation constants for binding these ligands to the query proteins. To see if these values were consistent with those found for the natural ligand-protein interactions, three ligands were chosen from Table 2 based on high (1KY), medium (AMP) and low (ADP) affinity to the query structures. Autodock Vina was then used to estimate the binding affinity for the ligands to the structures in their original PDB entries with these results.

- Ligand 1KY binds to PDB entry 1D6O [25] with a free energy of −6.8 kcal/mol and 10 μM dissociation constant.
- AMP binds to PDB entry 1K9Z [26] with a free energy of −8.5 and 0.60 μM dissociation constant.
- ADP binds to PDB entry 1PJH [27] with a free energy of −5.2 and 160 μM dissociation constant.

Based on these values, the range of dissociation constants for the ligand in Table 2 support biologically significant interactions that warrant further study. The binding of one ligand, nicotinamide adenine dinucleotide phosphate (NAP), to the query structure 4GHB [28] is further explored in Figure 3A and Figure 3B. This is a medium affinity case when binding NAP to 1PNO [29], and AutoDock predicts bindings of NAP to 4GHB in the vicinity of residues TYR-57, ARG-91 and TYR-188 as predicted, but also at two other nearby binding sites as shown in Figure 3B. The best affinity for the site involving TYR-57, ARG-91 and TYR-188 was −6,9 kcal/mol (K_d = 8,8 μM) achieved with the seventh of twenty models, while a better affinity of −7.6 kcal/mol (K_d = 2.7 μM) was found at a site more than 11 Å distant from the first model, and a credible affinity of −6.6 kcal/mol (K_d = 15 μM) at a third remote site. Thus, while the predicted function might be confirmed in future experiments, the multiple sites, the lack of specificity in binding orientation, and the pore-like conformation of the quaternary structure of the 4GHB dodecamer suggest that another possibility to explore is transmembrane transport of NAP [L.C. Andrews, private communication].

With these fifteen structures, ProMOL provided encouraging leads for exploring the function of these structures, while the other programs did not provide testable hypotheses.

Conflicting Function Assignment

Table 3 contains thirty structures, which gave promising alignments from all four programs. The noteworthy feature is that results from BLAST, Pfam and Dali were consistent, but ProMOL gave distinctly different results. In all but one case, there is poor agreement even to the first digit of the EC numbers.

Because Dali, BLAST and Pfam all suggested that PDB entry 1K77 [30] is a xylose isomerase or an epimerase with a 4-digit EC number 5.3.1.22, while ProMOL suggested EC number 3.1.1.29, we were particularly interested in the alignments of 1K77 in Table 4. The alignment is also shown in Figure 4. Based on the results in the other programs, ProMOL was used to compare 1K77 against all of our EC 5 motif templates. ProMOL revealed an alignment between 1K77 and one EC5 motif template: 1D6O, a peptidylprolyl isomerase EC 5.2.1.8. However, the RMSD values were greater than the cutoff values that were deemed reliable for alignments in ProMOL (non-hydrogen atoms/ C_α / C_α + C_β of 3.04/3.32/2.97 Å).

The importance of visual comparison—The ProMOL three-residue alignment between 1K77 (query structure) and 2PTH (motif template) [31] is shown in Figure 4A. 2PTH is a peptidyl-tRNA hydrolase with an EC number of 3.1.1.29. The RMSD value for C_α of the aligned residues is 1.18 Å and the visual alignment is reasonably good, with the three aligned residues having the side chains in similar orientations. The alignment of 1IUY [32] and 1NHC [33] shown in Figure 4B had similar RMSD values to the alignment of 1K77 and 2PTH, but the alignment of 1K77 and 2PTH is significantly better, based on visual inspection.

In another case, the alignment of 2KFL [34] and 1MOQ [35] (glucosamine 6-phosphate synthase; EC 2.6.1.16) yielded good RMSD values for an alignment of three of four residues (all non-hydrogen atoms/ C_α / C_α + C_β were 0.34 / 0.28 / 0.29 Å, respectively), but the visual alignment was much less convincing. BLAST, Dali and Pfam all predict that 2KFL is a prion protein. This is strongly supported by the Dali alignment (Figure 5) of 2KFL with 1QM2 [36] (a prion protein fragment), supporting the function proposed in Dali over the function proposed in ProMOL.

The proposed functions from Table 3 are clearly not as promising as those in Table 1 and Figure 2. Visual inspection is essential in these cases before deciding on next steps and the results found in ProMOL need to be carefully scrutinized and compared to the results found with the other programs before deciding the best approach for *in vitro* characterization.

Divergent Results with the Four Programs

Table 4 lists seven proteins that gave grouped results. For 1R3D [37], ProMOL and Pfam suggest a hydrolase, while BLAST and Dali suggest a 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC) synthase EC 4.2.99.2. Likewise for 2FBM [38], ProMOL and Pfam agree on lyase activity (EC 4), while BLAST and Dali place the structure under the transferase EC number 2. For two structures (2I1S [39] and 3KK4 [40]), the only agreement was between BLAST and Pfam. Only ProMOL and Pfam proposed functions for 3HFQ [41]; this is discussed in more detail below and in Figure 6. In the case of 3Q9D [42], there was no agreement among the programs and in the final case (4EZI [43]), ProMOL and Dali gave similar results.

In one intriguing case from Table 4, PDB entry 3HFQ aligns very well with chain A of motif template 1JOF [46], an isomerase with EC number of 5.5.1.5, with a C_α RMSD value of 0.56 Å. The initial ProMOL alignment between 3HFQ and 1JOF lacked the second arginine residue in 3HFQ. Alignment of the two sequences with Clustal Omega [47, 48] revealed that the 3HFQ contained ARG259, which aligned with ARG274 in the sequence of 1JOF. When the precision factor in ProMOL was relaxed from 1.0 to 1.1, ARG259 in 3HFQ aligned very closely with ARG274 in 1JOF (Figure 6).

While the Protein Data Bank characterizes 3HFQ as a protein of unknown function, it labels the molecule as “uncharacterized protein Ip_2219,” with an EC number 3.1.1.31, based on sequence alignment [49]. BLAST and Dali did not suggest a function for 3HFQ, but Pfam agreed with the PDB assignment in its suggestion that 3HFQ is a member of the 7-bladed beta-propeller lactonase family (EC 3.1.1.31). The Gene Ontology project has also placed 3HFQ under the EC number 3.1.1.31 [50]. It should be noted that InterPro [51] includes the lactonases (EC 3.1.1.31) and muconate lactonizing enzymes (EC 5.5.1.5) in the same entry as 7-bladed beta-propeller fold, IPR019405. Furthermore, of the ten structures in the PDB with EC 3.1.1.31, only 3HFQ and 1RI6 [52] demonstrated alignment with 1JOF (EC 5.5.1.5). The *in vitro* study of 3HFQ should help to resolve the correct EC number.

A manual BLASTP alignment of 3HFQ versus 1JOF provides insight into the lack of BLAST search results for 3HFQ, yielding poor alignment (21% identity) between the overall sequences. The ProMOL 1JOF motif active site residues align in BLASTP with the proposed active site residues in 3HFQ (Figure 6C, bold text), in runs of one to two sequence-aligned residues. This is a compelling example of the value of structural alignment.

Conclusions

With its current library of motifs, ProMOL has shown value in a significant, but limited, range of applications. The limitations can be addressed, in part, by extending the size of the motif library to cover larger motifs, such as the chromodomain, and more types of motifs, such as metal-ion-containing motifs. However, it will always have difficulty in identifying function based on global structure. When a ProMOL functional characterization is consistent with the BLAST, Pfam and Dali characterizations, as seen for the structures in Table 1, it is reasonable to assume that the expense of verification or rebuttal in the wet lab is justified.

The structures in Table 2 for which ProMOL found active site structural homology, but whose peptide sequence alignments did not yield significant results, require investigations into the possibility of convergent evolution. When a ProMOL functional characterization differs from the unanimous conclusions of the more global methods, this suggests that wet lab tests must do more than simply verify or refute a hypothetical characterization, because the possibility of multiple functions within a single protein or a sub-function intrinsic to the overall function of a protein must be considered. Table 3 outlines thirty such results. The structures in Table 4, for which there are conflicting results among ProMOL, BLAST, Pfam and Dali also require further, careful investigation.

ProMOL is a promising tool to determine the function of uncharacterized proteins, particularly in concert with other sequence and global alignment tools. ProMOL provides useful information because of its unique characterization approach in which it evaluates the three-dimensional alignment of active site residues. However, without additional *in silico* and *in vitro* structural and functional protein analysis data, ProMOL is not a sufficient tool to explicitly determine protein function. There are constant updates to the program, which serve to improve its accuracy and reliability. Nevertheless, due to the ambiguous nature of enzymatic active sites, it is crucial to further investigate any “good hit” generated by ProMOL.

For the cases in which ProMOL results are in strong agreement with BLAST, Pfam, and Dali results, there is a high probability of being able to determine protein function experimentally. The fact that four different *in silico* approaches for protein analysis yielded similar conclusions means that those query macromolecules possess a significant number of the major characteristics of motifs from those corresponding classifications.

For most of the query structures, ProMOL generated a result that differed from results produced by BLAST, Pfam, and Dali. In some of these cases (Table 2), BLAST, Pfam and Dali collectively returned insignificant or no results. In others (Table 3), the latter three programs all agreed. In a third set of predictions (Table 4), individual results were incompatible. In all three of these instances it is difficult to draw conclusions, and further investigation into the bases for these discrepancies is warranted. The results for ligand binding with Autodock Vina shown in Table 2 offer significant guidance for substrate selection for future *in vitro* characterization studies.

By building on and extending current best practices, the methodology we are following shows promise of providing both more specificity and more reliability than existing approaches for ascribing function to proteins for which functions are not yet known experimentally. In addition to helping identify the functions of the large number of proteins of unknown function, this approach seems likely to help achieve a clearer understanding of structure-function relationships and to improve, codify and simplify existing laboratory practice in this important aspect of health-related research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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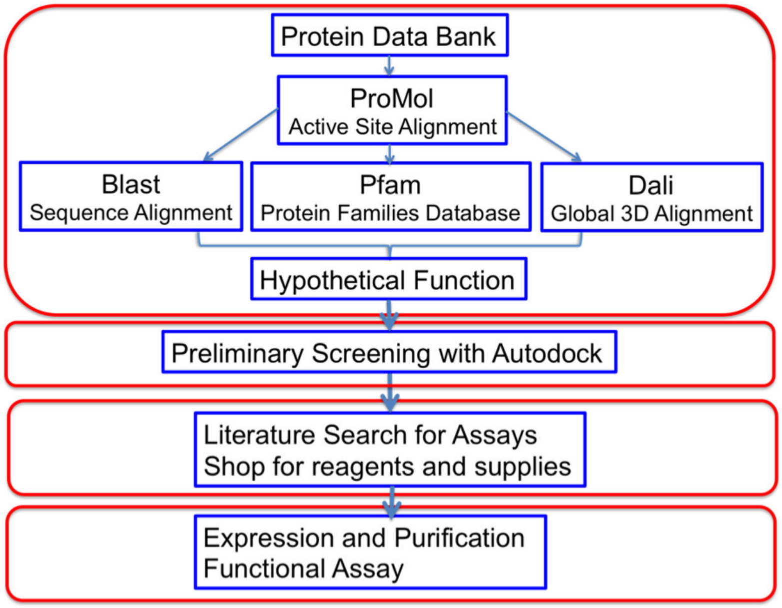

Protein Data Bank
ProMol
Active Site Alignment
Blast
Sequence Alignment
Pfam
Protein Families Database
Dali
Global 3D Alignment
Hypothetical Function
Preliminary Screening with Autodock
Literature Search for Assays
Shop for reagents and supplies
Expression and Purification
Functional Assay

Figure 1.

Flowchart of the process used for characterization of proteins of “unknown function”. Although *in silico* characterization methods and results in the top box are the focus of this report, additional *in silico* (the second box), *in biblio* (third box) and *in vitro* steps (fourth box) are also illustrated.

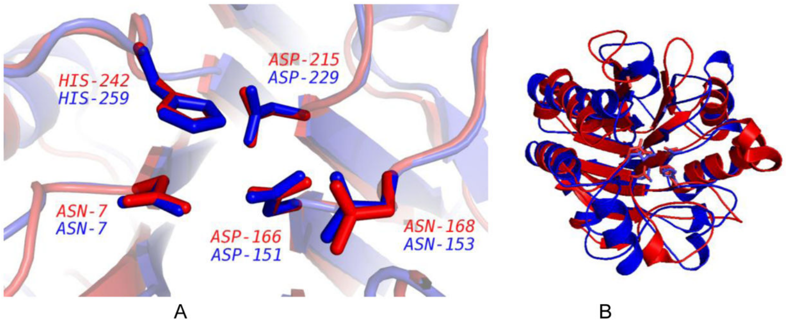


Figure 2.

A. The five residues aligned by ProMOL are drawn for 3L1W (query/red) and 1AKO (motif template/blue). B. The superposition of 3L1W (red) and 1AKO (blue) was generated by Dali and visualized in PyMOL.

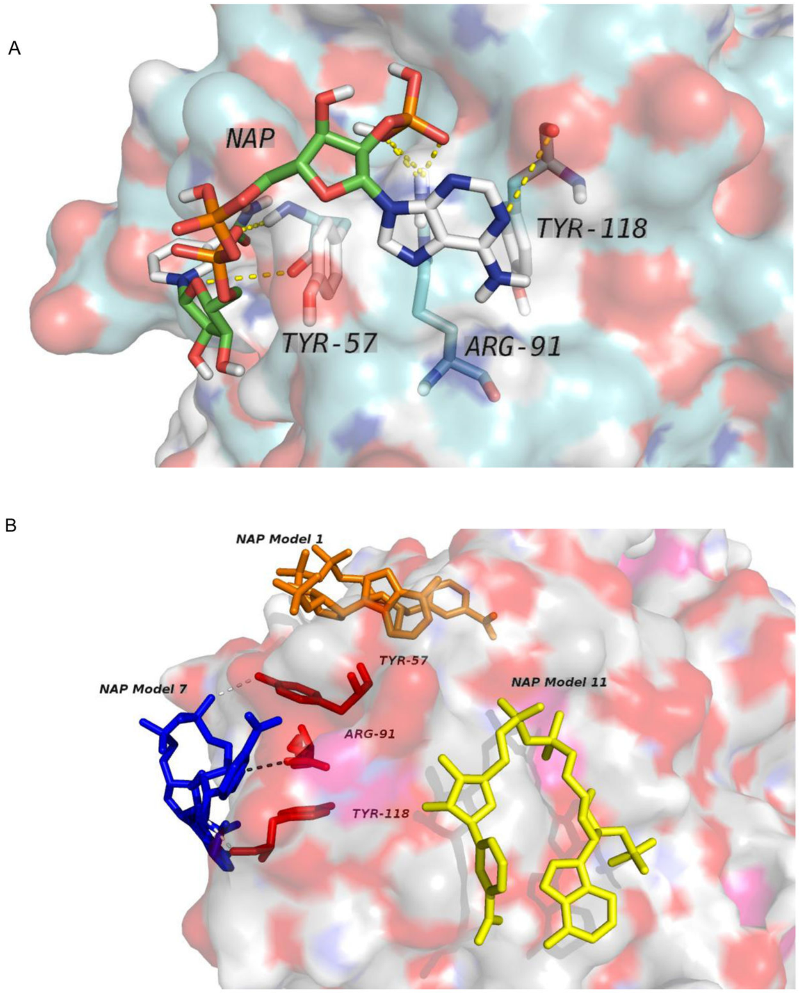


Figure 3.

Predicted ligand binding. A. The binding of nicotinamide adenine dinucleotide phosphate (NAP) to three residues in the proposed active site of PDB entry 4GHB was predicted by AutoDock Vina at a binding site involving TYR-57, ARG-91 and TYR-188 as predicted, but also (B) at two or more additional adjacent binding sites not involving those residues.

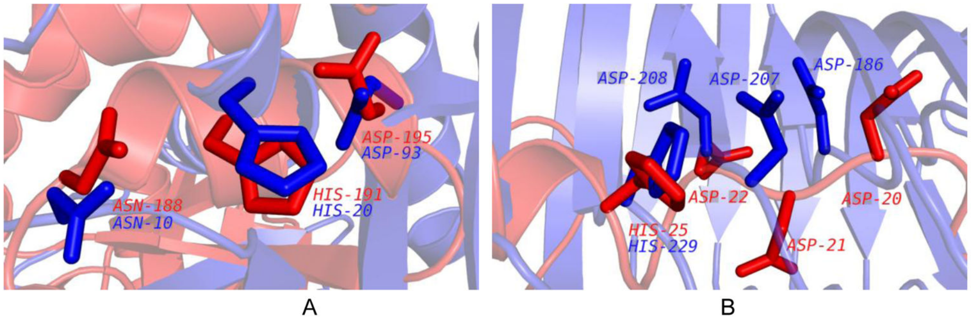


Figure 4.

The significance of visual alignment. A. The aligned residues are shown for 1K77 (query/red; ASN188, HIS191, and ASP195) and for 2PTH (motif template/blue; ASN10, HIS20, and ASP93). The RMSD values for all non-hydrogen atoms / C_α / C_α + C_β were 1.91 / 1.18 / 1.30 Å, respectively. B. The aligned residues shown for 1IUY (query/red; ASP20, ASP21, ASP22, and HIS25 and 1NHC (motif template/blue; ASP186, ASP207, ASP208, and HIS229). The RMSD values for all non-hydrogen atoms / C_α / C_α + C_β were 1.93 / 1.64 / 1.56 Å, respectively.

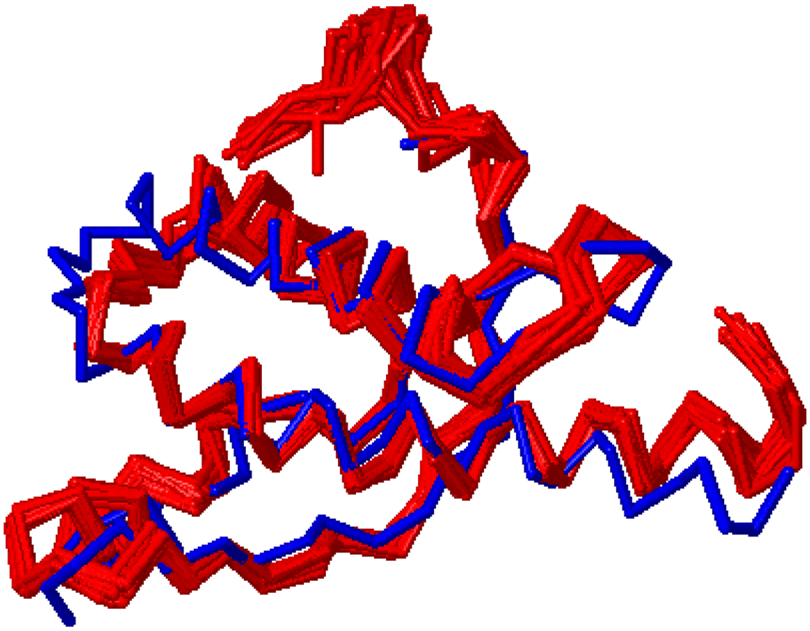
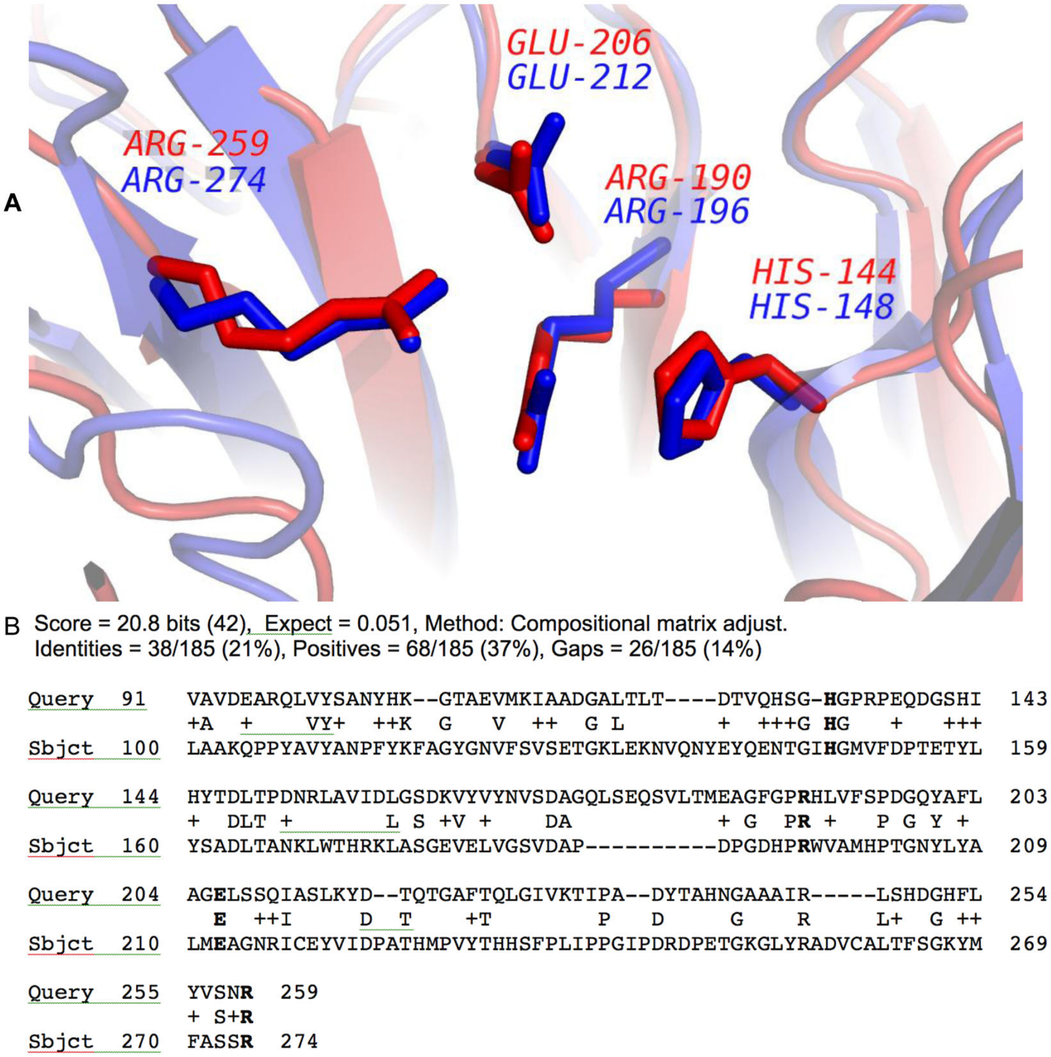


Figure 5.

Superposition of 2KFL (red) and 1QM2 (human prion protein fragment 121 – 230, blue). The image was generated from the Dali website.



Score = 20.8 bits (42), Expect = 0.051, Method: Compositional matrix adjust.
Identities = 38/185 (21%), Positives = 68/185 (37%), Gaps = 26/185 (14%)

```
Query  91   VAVDEARQLVYSANYHK--GTAEVMKIAADGALTLT----DTVQHSG-HGPRPEQDGSHI  143
            +A  +    VY+  ++K  G   V  ++  G L       +  +++G HG   +   +++
Sbjct  100  LAAKQPPYAVYANPFYKFAGYGNVFSVSETGKLEKNVQNYEYQENTGIHGMVFDPTETYL  159

Query  144  HYTDLTPDNRLAVIDLGSDKVYVYNVSDAGQLSEQSVLTMEAGFGPRHLVFSPDGQYAFL  203
            +  DLT +       L S +V +    DA           + G  PR +   P G Y +
Sbjct  160  YSADLTANKLWTHRKLASGEVELVGSVDAP----------DPGDHPRWVAMHPTGNYLYA  209

Query  204  AGELSSQIASLKYD--TQTGAFTQLGIVKTIPA--DYTAHNGAAAIR-----LSHDGHFL  254
              E  ++I     D  T    +T        P   D     G    R     L+  G ++
Sbjct  210  LMEAGNRICEYVIDPATHMPVYTHHSFPLIPPGIPDRDPETGKGLYRADVCALTFSGKYM  269

Query  255  YVSNR  259
            + S+R
Sbjct  270  FASSR  274
```

Figure 6.

Four-residue alignment of 3HFQ (query/red) with 1JOF (motif template/blue). A. The order of residues and the spacing between residues in the sequences of both structures is nearly identical. B. The sequences of 3HFQ and 1JOF were aligned with BLASTP and the largest alignment is displayed. The catalytic residues shown in 6A are highlighted in bold.

Table 1

Cases in which ProMOL, BLAST, Pfam, and Dali all yielded similar functional annotations for a given query. The items in the column entitled, “Function Assigned by ProMOL”, were determined based on the most general classification of the motif with which the query structure was aligned. The PDB IDs of the motif templates that yielded these alignments can be found in Table 1S. There are thirteen query structures to which all four programs assigned functions that were under the same top-level EC number. There are more alignments (eighteen) than structures (thirteen) because ProMOL returned multiple alignments for several query structures. Fourteen of eighteen alignments in ProMOL consisted of identical active site residue matches between the query and the motif template.

Query ID	Function Assigned by ProMOL	EC Number Assigned by ProMOL	Common function from Dali, BLAST, Pfam	EC Number Assigned by Dali, BLAST, Pfam
1MK4[a]	transferase	2.4.2.31	acetyltransferase	2.3
2AQW	lyase	4.1.1.23	orotidine-5′-phosphate decarboxylase	4.1.1.23
2I3D	hydrolase	3.7.1.8 or 3.4.11.5	alpha/beta hydrolase	3
2O14	hydrolase	3.1.1.47 or 3.4.11.5	esterase or lipase	3.1
2PW6	oxidoreductase	1.11.1.6	dioxygenase	1.13.11 or 1.14
2R8B	hydrolase	3.4.11.5 or 3.1.1.3	hydrolase or esterase	3 or 3.1.1
2YYS	hydrolase	3.7.1.9	hydrolase or proline iminopeptidase	3 or 3.4.11.5
3CBW	hydrolase	3.2.1.31	beta-mannanase or glycosyl hydrolase family 26	3.2
3DS8	hydrolase	3.1.1.3	alpha/beta hydrolase or esterase	3
3H04	hydrolase	3.4.11.5 or 3.7.1.8 or 3.4.21.26	alpha/beta hydrolase or esterase	3 or 3.1.1
3L1W	hydrolase	3.1.11.2	endonuclease/exonuclease	2 or 3.1
4DIU	hydrolase	3.4.11.5	hydrolase or carboxylesterase or lipase	3 or 3.1.1
4Q7Q	hydrolase	3.1.1.47	GDSL-like lipase	3.1.1

[a] The citations for all of the PDB structures in this manuscript can be found in Table 1S.

Table 2

ProMOL assigned functions to structures for which BLAST, Pfam, and Dali did not suggest an assignment. There were fifteen instances in which ProMOL suggested a function for a structure, while the other three programs did not. RMSD values were calculated for all non-hydrogen atoms. Fourteen of fifteen alignments in ProMOL consisted of identical active site residue matches between the query and the motif template.

Query ID	Motif ID	EC Number of Motif	RMSD: All (Å)	Function Assigned by ProMol	Ligand[a]	ΔG (kcal/mol)[b]	K_d (μM)
1OQ1	1E2T	2.3.1.118	1.94	transferase	KH2	−5.8	57
2DBI	1BP2	3.1.1.4	2.29	hydrolase	8IN	−6.1	34
2G6T	1NAA	1.1.99.18	2.13	oxidoreductase	ABL	−6.1	34
2GFQ	1D6O	5.2.1.8	2.44	isomerase	1KY	−10.2	0.034
2P0O	1K9Z	3.1.3.7	0.93	hydrolase	AMP	−7.2	5.3
2P0V	1AAM	2.6.1.1	1.96	transferase	CBA	−6.8	10
2PK7	1PJH	5.3.3.8	1.64	isomerase	ADP	−5.1	180
2Q4K	1OD1	3.4.23.22	2.08	hydrolase	0EO	−6.1	34
2XU2	1BMT	2.1.1.13	1.52	transferase	C2F	−5.7	67
3D19	1HY3	2.8.2.4	2.02	transferase	EST	−9.5	0.11
3L6I	1C4X	3.7.1.8	2.14	hydrolase	22J	−4.8	305
3M3I	4HOH	3.1.27.3	2.69	hydrolase	2GP	−6.7	12
3NLC	1BMT	2.1.1.13	1.54	transferase	SAH	−7.2	5.3
3RBY	1BK7	3.1.27.1	2.15	hydrolase	5GP	−6.5	17
4GHB	1PNO	1.6.1.2	1.75	oxidoreductase	NAP	−8.1	1.2

[a] The abbreviations are the names listed in the PDB for the following ligands:

KH2: 3-(1-methylpiperidinium-1-yl)propane-1-sulfonate

8IN: [3-(1-benzyl-3-carbamoylmethyl-2-methyl-1h-indol-5-yloxy)-propyl-]-phosphonic acid

ABL: 5-amino-5-deoxy-cellobiono-1,5-lactam

1KY: 6-({(1S,5R)-3-[2-(3,4-dimethoxyphenoxy)ethyl]-2-oxo-3,9-diazabicyclo[3.3.1]non-9-yl}sulfonyl)-1,3-benzothiazol-2(3H)-one

AMP: adenosine 5′-monophosphate

CBA: N-pyridoxyl-2,3-dhydroxyaspartic acid-5-monophosphate

ADP: adenosine 5′-diphosphate

0EO: (2S)-2-[[(3S,4S)-5-cyclohexyl-4-[[(4S,5S)-5-[(2-methylpropan-2-yl)oxycarbonylamino]-4-oxidanyl-6-phenyl-hexanoyl]amino]-3-oxidanyl-pentanoyl]amino]-4-methyl-pentanoic acid

C2F: 5-methyl-5,6,7,8-tetrahydrofolic acid

EST: estradiol

22J: (3E,5R)-5-fluoro-6-(2-fluorophenyl)-2,6-dioxohex-3-enoic acid

2GP: guanosine-2′-monophosphate

SAH: S-adenosyl-L-homocysteine

5GP: guanosine-5′-monophosphate

NAP: nicotinamide adenine dinucleotide phosphate

[b] The binding energy as computed by Autodock Vina [16] was converted to a dissociation constant with the formula: K_d=exp(−ΔG/(R*T)) [http://autodock.scripps.edu/faqs-help/faq/how-autodock-4-converts-binding-energy-kcal-mol-into-ki]

Table 3

Query structures for which BLAST, Pfam, and Dali provided a consensus EC number while ProMOL reported a conflicting result. There were thirty structures for which all four programs gave promising results. These are the instances in which BLAST, Pfam, and Dali all returned similar results, but ProMOL did not. Twenty-eight of thirty-seven alignments in ProMOL consisted of identical active site residue matches between the query and the motif template.

Query ID	Function Assigned by ProMOL	EC Number Assigned by ProMOL	Common function from Dali, BLAST, Pfam	EC Number Assigned by Dali, BLAST, Pfam
1IUY	hydrolase	3.2.1.15	cullin family (ligase)	6
1K77	hydrolase	3.1.1.29	xylose isomerase; epimerase	5.3.1.22
1NOG	oxidoreductase	1.1.1.158	adenosyltransferase	2.5
1VQW	lyase	4.2.1.24	flavin monooxygenase	1.14
1YEM	oxidoreductase	1.1.1.85	adenylate cyclase	4.6.1.1
1YEY	hydrolase	3.1.3.7 or 3.1.21.1	mandelate racemase	5.1.2.2
1YRE	hydrolase	3.5.1.5	acetyltransferase	2.3
1YX3	hydrolase	3.2.1.14	sulfite reductase	1.8
1Z40	transferase	2.7.11.13	apical membrane antigen 1	N/A[a]
2DAF	hydrolase	3.7.1.8	ubiquitin-like domain	1, 2, or 3
2DC4	isomerase	5.4.99.5	adenylate cyclase	4.6.1.1
2DEC	hydrolase	3.5.2.6	phosphosugar isomerase	5.3
2F06	oxidoreductase	1.5.1.34	aspartokinase	2.7.2.4
2GGE	ligase	6.3.2.9	isomerase; mandelate racemase	5 or 5.1.2.2
2KFL	transferase	2.6.1.16	prion protein	N/A[a]
2Q4D	hydrolase	3.7.1.9	lysine decarboxylase	4.1.1.18
2R85	hydrolase	3.5.1.11	formate-phosphoribosylaminoimidazole carboxamide ligase	6.3
2YWO	transferase	2.3.1.118	thiol-disulfide isomerase; alkyl hydroperoxide reductase	5.3.1.11
2Z0J	oxidoreductase	1.1.1.158	2-phosphosulpholactate phosphatase	3
2Z6V	isomerase	5.3.3.10	sulfotransferase	2.8
2ZBV	hydrolase	3.2.1.45	S-adenosyl-1-methionine hydroxide adenosyltransferase	2.5.1
3EC6	isomerase	5.3.3.1	general stress protein; pyridoxamine 5′-phosphate oxidase	N/A or 1
3E8X	isomerase	5.1.3.20	oxidoreductase or NADP binding	1

Query ID	Function Assigned by ProMOL	EC Number Assigned by ProMOL	Common function from Dali, BLAST, Pfam	EC Number Assigned by Dali, BLAST, Pfam
3FLJ	hydrolase	3.1.3.7	SnoaL_2 domain or steroid delta-isomerase	5
3NRN	transferase	2.1.1.184	oxidoreductase	1
3RE2	transferase	2.3.3.1	menin	N/A[a]
3S5E	lyase	4.2.1.24	mitochondrial frataxin	N/A[a]
3TB2	hydrolase	3.1.3.7	1-cys peroxidoxin	N/A[a]
4ILS	hydrolase	3.8.1.5	alanine racemase	5.1.1.1
4J10	hydrolase	3.5.1.77	secreted protein	N/A[a]

[a] Dali, BLAST and Pfam all agreed on the putative nonenzymatic functions for these protein structures; N/A indicates that no EC number is provided in these cases.

Table 4

Divergent Results. It is impossible to assign these structures to consensus EC numbers based on ProMOL, BLAST, Pfam, and Dali results. All programs generated promising, yet varying, results. Six of seven alignments in ProMOL consisted of identical active site residue matches between the query and the motif template.

Query	ProMOL: Assigned Function and EC	BLAST: Assigned Function and EC	Pfam: Assigned Function and EC	Dali: Assigned Function and EC
1R3D	Hydrolase (3.1.1.3)	SHCHC synthase (4.2.99.20)	alpha/beta hydrolase (3)	SHCHC synthase[a] (4.2.99.20)
2FBM[b]	lyase (4.2.1.24)	Y-like chromo domain (2.3.1.48)	enoyl-CoA hydratase (4.2.1.17) or isomerase (5.3.3.8)	Y-like chromadomain[b] (2.3.1.48)
2I1S	isomerase (5.3.1.5)	plasmid pRiA4b ORF-3 family protein	plasmid pRiA4b ORF-3 family protein	No significant results
3KK4	hydrolase (3.1.3.7)	RHH-4 superfamily and COG4321	RHH_4 super- family	No significant results
3HFQ	Isomerase (5.5.1.5)	No significant results	7-bladed beta- propeller lactonase family (EC 3.1.1.31)	No significant results
3Q9D	hydrolase (3.2.2.1)	various hypothetical/ uncharacterized proteins and CT584 protein	no significant results	CT584 protein
4EZI	hydrolase (3.4)	various hypothetical proteins	no significant results	esterase or lipase (3)

[a] For PDB entry 1R3D, the catalytic triad SER-HIS-ASP is typical of both alpha/beta hydrolases and SHCHC synthases [44].

[b] For PDB entry 2FBM, ProMOL does not yet manage motifs of the size of the chromodomain. Regarding PDB entry 2FBM, many members of the crotonase (or enoyl-CoA hydratase) family have similar structures, but differing functions as they are from separate enzyme classes [45]. *In vitro* testing of these cases might prove the most interesting in establishing the value of each ProMOL functional assignment relative to BLAST, Pfam and Dali.