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The Application of FAST-NMR for the Identification of Novel Drug Discovery Targets

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Abstract

The continued success of genome sequencing projects has resulted in a wealth of information, but 40-50% of identified genes correspond to hypothetical proteins or proteins of unknown function. The **F**unctional **A**nnotation **S**creening **T**echnology by NMR (FAST-NMR) screen was developed to assign a biological function for these unannotated proteins with a structure solved by the Protein Structure Initiative. FAST-NMR is based on the premise that a biological function can be described by a similarity in binding sites and ligand interactions with proteins of known function. The resulting co-structure and functional assignment may provide a starting point for a drug discovery effort.

Keywords

NMR High-throughput Screens; NMR; Structural Biology; Protein Structure Initiative; Functional Genomics; Hypothetical Proteins

The completion of the human genome project is spurring tremendous progress in cell biology, development, evolution and physiology [1]. The expanding number of protein structures emerging from the Protein Structure Initiative (PSI) is contributing to these advancements [2]. As of January 2007, the sequencing of 607 genomes has been completed with 1676 ongoing projects. Also, nearly 2,500 protein structures have been solved by PSI [3,4]. Drug discovery is benefiting from these successes through the identification of novel therapeutic targets and the development of new tools to optimize chemical leads [5-7]. As an example, the identification of novel anti-infectious targets may aid in avoiding common mechanisms of resistance and extend the lifetime of new antibiotics [8,9].

An underlying challenge to capitalizing on genome sequencing efforts is the abundance of hypothetical proteins, proteins that lack a functional annotation. Our recent analysis of various bacterial genomes from the August 2007 Gold release shows that, even with improved computational methods, approximately 40% of bacterial proteins have not been assigned to a functional category (Figure 1) [3]. There are more than 11,000 proteins from the ten bacterial organisms listed in Figure 1 that lack a functional annotation. Considering this list is only from a small segment of currently sequenced genomes, the prospect of obtaining experimental

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Teaser: FAST-NMR is a high-throughput method to determine biological function of hypothetical proteins identified from the Protein Structure Initiative as a means to further biological knowledge and drug discovery efforts.

functional information for all hypothetical proteins identified from completed and ongoing sequencing efforts is a daunting proposition. Valuable information is hidden among this multitude of unannotated proteins that could be associated with cell viability, biofilm formation, infection, and pathogenesis. These proteins may provide key information for developing new antibiotics, where drug discovery efforts would benefit greatly from new functional annotations methodology.

Most high-throughput experimental methods to assign function have focused primarily on generating knockout libraries to analyze cell phenotypes, monitoring changes in gene expression or determining protein interaction maps [10-12]. These methods generally do not provide functional information for a specific protein without additional detailed bioinformatics [13,14]. Global sequence similarity is routinely used to infer the function of hypothetical proteins, despite analysis that suggest error rates are as high as 30% [15,16]. Conversely, amino-acid residues associated with the active-sites and biological activities of proteins are stable evolutionarily relative to the remainder of the protein's sequence and provide an alternative approach for functional annotation [17,18]. A basic definition of biological function is derived from a protein's interaction with small molecules and other biomolecules. Thus, the identification of functional ligand(s), an active site and a corresponding protein-ligand co-structure is instrumental to defining a function for a hypothetical protein. The comparison and prediction of ligand binding sites from both structural and sequence information is a proven approach for functional assignments of proteins [19]; however, these predictions may lead to ambiguous or incorrect annotations [20,21]. A combination of experimental protein-ligand binding data with bioinformatic analysis will minimize the uncertainties commonly associated with pure computational approaches.

FAST-NMR (**F**unctional **A**nnotation **S**creening **T**echnology by NMR) provides functional information for hypothetical proteins by experimentally characterizing and analyzing ligand binding sites [22]. Once the functional ligands [23] are identified and the binding site is located, a co-structure is obtained. A functional assignment is deduced by comparing the ligand-defined active-site from FAST-NMR to a database of protein-ligand binding sites for proteins of known function using CPASS (**C**omparison of **P**rotein **A**ctive-**S**ite **S**tructures) [24]. The information obtained from FAST-NMR furthers our understanding of the basic biological role of hypothetical proteins and provides a potential starting point for drug discovery. A summary of some common applications for the functional annotation of proteins of unknown proteins and a comparison to our FAST-NMR method are listed in Table 1.

The FAST-NMR Method

FAST-NMR was developed to assign biological functions to hypothetical protein structures solved by PSI. Recent statistical analysis indicates that ~20-50% of protein structures determined by PSI may be amenable to analysis by NMR [25,26]. The Protein Structure Database (PDB) currently contains ~2,200 proteins of unknown function [27]. These proteins tend to be “orphaned” from any further functional analysis because of a complete absence of information to guide a research project [28-31]. These orphaned proteins are ideal targets for analysis by FAST-NMR. An assigned ^{1}H-^{15}N Heteronuclear single quantum coherence (HSQC) NMR spectrum and a corresponding structure for a protein of unknown function are the primary requirements for the FAST-NMR methodology. In general, high-resolution NMR structures and assignments can be routinely obtained for proteins <25 kDa using standard ^{13}C and ^{15}N protein labeling techniques [32,33]. This molecular-weight upper limit may be extended by upwards of 900 kDa [34-40] by the application of deuterium labeling, specific methyl labeling and Transverse Relaxation-Optimized NMR Spectroscopy (TROSY)-based experiments [41-43].

The FAST-NMR assay applies a tired approach to screening, where an overview of the methodology is illustrated in Figure 2. The first 1D ^1H line-broadening (LB) experiments are amenable for screening a large number of compounds in a relatively short time (< 10 minutes/sample) and requiring a minimal amount of unlabeled protein material (< 0.1 mgs/sample). Only positive "hits" from the LB experiments are further screened in 2D ^1H-^{15}N HSQC spectra. In this manner, the tiered approach minimizes resources and increases throughput by funneling only the most promising candidates forward to the more resource intensive 2D ^1H-^{15}N HSQC experiments.

In the first LB experiment, the formation of a protein-ligand complex can be determined by monitoring changes in the ligand's spectrum. Binding of the ligand to the protein causes line width broadening that may result in the complete disappearance of the ligand's NMR peak(s). This is caused by the large differences in molecular-weight and correlation time (τ_c, time it takes the molecule to rotate one radian) between the protein and small molecule. In the second NMR experiment, changes in the protein's NMR spectrum are followed to further confirm a specific interaction and identify a potential binding site. Ligand binding causes local environmental changes in the protein resulting in the observation of chemical shift perturbations (CSPs) in the 2D ^1H-^{15}N HSQC spectrum. Since each peak in the HSQC spectrum has been sequentially assigned to a specific amino acid in the protein's sequence, the CSPs can be mapped onto the surface of the protein. A consensus clustering of CSPs identifies the location of the ligand binding site. A lack of CSPs or a random distribution of CSPs over the protein's surface indicates non-specific binding of the ligand to the protein.

A rapid protein-ligand co-structure is determined by combining the experimental CSPs with molecular modeling. AutoDock [44] has been demonstrated to outperform two other well-known docking tools, FlexX and DOCK [45] in a virtual screen, and is currently the most cited of molecular modeling applications [46]. The experimental CSPs define a grid used by AutoDock to guide the ligand docking into the NMR defined binding site. Our AutoDockFilter (ADF) program is then used to filter the AutoDock conformers and select a pose that best fits the CSPs. In general, amino acid residues with the largest CSPs are expected to be closer to the bound ligand relative to residues with smaller CSPs. The best-structure is then used to determine a ligand-defined binding site for CPASS analysis. CPASS identifies ligand-defined binding sites for proteins of known function from a PDB derived database that best matches the sequence and structural details of the ligand-defined binding site determined by FAST-NMR. A similarity between these ligand-defined binding sites infers a function that can be assigned to the hypothetical protein.

Functional Chemical Library

A critical component of the FAST-NMR methodology is the functional chemical library [47] (Figure 3). The library contains compounds with demonstrated protein affinity and, as completely as possible, covers the diversity of biological activity. The library is screened for binders by NMR to probe for protein function and includes known drugs, inhibitors and protein substrates that target a diverse set of protein functions covering a range of structural chemical classes. This list includes amino-acids, carbohydrates, co-factors, fatty-acids, hormones, metabolites, neurotransmitters, nucleic acids and vitamins. Importantly, the distribution of chemical and physical properties for the compounds in the functional library is similar to drug-like molecules (MW < 500 Da, number of heteroatoms < 10, cLogP < 5, and number of rings < 2). This indicates that compounds identified as binders in a FAST-NMR screen may also be useful starting points for structure-based drug design, if the hypothetical protein is identified as a valuable therapeutic target.

The design of the functional chemical library was optimized for screening by NMR. The compounds are soluble in water, do not aggregate, precipitate or react in mixtures and have unique NMR resonances for ready identification that avoids deconvolution. The selection process for the compounds in the functional library was extensive and based on a number of criteria: known biological activity, the existence of a co-structure in the Protein Database (PDB) [48], the likelihood of aqueous solubility (≥ 100μM), purity (≥90%), commercial availability (≥ 5mg) and cost (≤ $32). Each of the compounds are dissolved in D_6-DMSO or D_2O and stored in 96-well plates in a dessicator in a −80°C freezer. Compounds are screened in mixtures to further increase throughput while minimizing resources. The mixtures were designed to contain 3-4 compounds based on our statistical analysis of the optimal mixture size for NMR screens [49].

CPASS

CPASS incorporates a computer program with a structural database to compare ligand binding sites and provide a putative function for hypothetical proteins screened by FAST-NMR [24]. CPASS is a structure-based functional annotation program that differs from a variety of 3D template or sequence-based annotation programs (for a review see Watson *et al.* (2005) [50]) routinely used to predict ligand binding sites and protein function. These programs attempt to *predict* the location of ligand binding sites using various sequence and structure heuristics. Instead, CPASS aligns *experimentally* determined ligand-defined binding sites from FAST-NMR and the PDB using sequence and structural descriptors. Simply, CPASS identifies matches between functionally relevant ligand-binding sites to leverage an annotation.

CPASS may also aid the development of selective chemical leads. Drug toxicity is a common cause of clinical failures [51], where this toxicity is associated with non-specific *in vivo* protein activity [52]. In practice, it is not possible to screen against every potential protein target that may bind a chemical lead. Instead, a small panel of homologous proteins is used in secondary assays to infer compound specificity. The proteins are generally selected based on global sequence similarity to the protein target of interest. Unfortunately, there are also other proteins that share a high similarity in the ligand binding site that may lack global sequence similarity. Our previous CPASS analysis of ATP binding proteins indicates a significant cluster of proteins with sequence similarity < 20% that had high CPASS similarity of > 40% [24]. Similarly, we identified two alanine racemases that share only an 8% sequence similarity, but had essentially identical PLP binding sites. Clearly, proteins that share high ligand binding site similarity, but lack global sequence similarity pose serious risks of causing toxic side-effects in clinical trials unless identified using applications like CPASS.

Protein-Ligand Database

The CPASS database is continuously updated from the PDB and contains proteins in complex with small molecules, peptides, and oligonucleotides. Proteins may bind one ligand, multiple ligands, or the same ligand more than once. Each unique ligand-binding site (< 80% sequence similarity, distinct ligand) is incorporated into the CPASS database. There are ~55,000 protein-ligand binding sites currently present in the PDB, where ~21,000 are unique. These ligand-defined binding sites include all the amino acids in the protein sequences that have at least one atom within 6 Å of any atom of the ligand. Both the structure coordinates and the sequence identity are then used in a comparison with ligand-defined binding sites from other proteins. The ligand structure is not included in the ligand-defined binding site, but is used to classify the type of binding site (i.e. ATP binding site, FAD binding site, etc).

Similarity Scoring Function

Although the CPASS program will allow the user to search binding-sites based on the type of ligand that defines the binding site, it is not required. The comparison can be made against all ligand-defined binding sites present in the CPASS database or any ligand-type subset. The CPASS scoring function is based on the simultaneous structure and sequential alignments of two ligand-defined binding sites. A BLOSUM62 probability function weighted by root-mean square distance (rmsd) is used to compare the similarity of spatially aligned residues:

$$S_{ab}=\sum_{i,j=1}^{i=n,j=m}\frac{d_{min}}{d_i}\left(e^{-\Delta rmsd_{i,j}}\right)^2 p_{i,j}$$

$$\Delta rmsd_{i,j}=\begin{cases} rmsd_{i,j} & rmsd_{i,j}>1\text{Å} \\ 0 & rmsd_{i,j}\le 1\text{Å} \end{cases}$$

Active site *a* contains *n* residues and is compared to active site *b* of *m* residues from the CPASS database. $p_{i,j}$ is the BLOSUM62 probability for replacement of amino acid *i* from active site *a* with residue *j* from active site *b*, $\Delta rmsd_{i,j}$ is the corrected root-mean-square-difference in the Cα positions between the residues *i* and *j*, and d_{min}/d_{i} is the ratio of the shortest distance to an atom in the ligand from any atom in the residue *i*. This last term minimizes boundary effects. Small structural changes may result in residues entering or leaving the 6Å cut-off used to define a ligand-defined binding site. This may result in relatively large changes in the scoring function due to modest structural fluctuations.

The similarities between the active sites are then calculated by:

$$S=S_{ab}/S_{aa}\times 100$$

where *S* is the similarity score, S_{ab} is the similarity score for the protein target against an active site from the CPASS database, and S_{aa} is the similarity of the active site compared to itself used for normalization. In effect, a percent similarity is determined based on how well the sequence and structures of the two ligand-binding sites overlap. The scoring function is not symmetrical since it depends on the size of the binding site.

CPASS Functional Prediction of Hypothetical Proteins

To illustrate further the utility of CPASS, a recent protein deposited in the PDB was chosen that only had a putative functional annotation. A human protein (PDB-ID 2PL3) was tentatively assigned as a probable ATP-dependent RNA helicase DDX10 and the structure contained a bound ADP molecule. CPASS analysis identified PDB-ID 2OXC as having the highest similarity (56.26%). Both proteins bind ADP and are hypothetical DEAD domains. The highest CPASS similarity score (50.30%) to a protein of known function was to PDB-ID 1XTJ, a DECD to DEAD mutation of human UAP56, which is also in complex with ADP [53]. Recently, the UAP56 protein has been shown experimentally to exhibit RNA-stimulated ATPase activity and ATP-dependent RNA helicase activity [54]. Thus, the CPASS analysis supports the prior putative assignments of hypothetical proteins 2PL3 and 2OXC as ATP-dependent RNA helicases. The top panel in Figure 4 shows the alignment of the ADP binding sites for the 2PL3 and 1XTJ structures. This figure clearly highlights the overall similarity in the structure and sequence alignments for the ADP binding sites.

A crystal structure of hypothetical protein PH1320 from *Pyrococcus horikoshii* OT3 was recently released by the PDB (PDB-ID 2E87). The protein is complexed to guanosine-5′-diphosphate (GDP), but completely lacks a functional assignment and a paper describing the structure has yet to be published. A CPASS analysis using only proteins complexed to GDP indicates hypothetical protein PH1320 has a very high similarity (70.47%) to an *Escherichia coli* elongation factor Der (PDB-ID 1MKY), an EngA homolog [55]. The bottom panel in figure 4 clearly demonstrates the high overall similarity in the structure and sequence alignments for the GDP binding sites between these two proteins. Hypothetical protein PH1320 shows CPASS similarity scores of 50-70% to EngB, EngC, EI-F2γ, EI-F5B, EF-Tu, EF-1α, EF-2 and EF-G, which are also members of the elongation factor super family. Hypothetical protein PH1320 also exhibits a slightly smaller similarity (50-60%) to Arf, Sar and Rab, members of the small GTPase super family that regulate a diverse range of cellular events [56]. Thus, the CPASS results suggest PH1320 is probably an elongation factor or potentially involved in GTP signal regulation similar to either Arf, Sar or Rab.

Functional Annotation of *Staphylococcus aureus* Protein SAV1430

Staphylococcus aureus protein SAV1430, a hypothetical protein of unknown function, was selected to demonstrate the FAST-NMR methodology (Figure 2). SAV1430 is a typical target of the NorthEast Structural Genomic Consortium (NESG) [29], where a structure was previously determined [57,58]. A Dali analysis suggested that SAV1430 has a similar topology to a ferredoxin-like fold, but the Z-score of < 3 was insignificant [59]. The only proteins that had any significant sequence homology to SAV1430 were other hypothetical proteins, so a reliable function could not be assigned based on structure homology alone.

O-phospho-L-tyrosine (pTyr) was identified as one of 21 compounds that exhibited line-broadening and chemical shift perturbations in the FAST-NMR screen with SAV1430. The other compounds are chemically similar to pTyr and were all shown to interact in a consensus binding-site that comprises residues I6-P10, T14-K16 and I61-V63. This binding site contains a shallow cleft on the SAV1430 surface surrounded by relatively flat structural features strongly suggestive of a protein-protein interaction site. A rapid structure of the pTyr-SAV1430 complex was determined using CSPs and AutoDock for CPASS analysis.

CPASS identified PDB ID 1oo4 as a significant hit (37% similarity), a Src SH2 domain complexed with a pTyr containing peptide. SH2 domains are typically part of multi-domain proteins involved in cell signaling and form a protein-protein complex with a kinase after phosphorylation of a tyrosine [60]. Phosphorylation of Ser, Thr and Tyr are also common mechanisms for regulating protein activity in bacteria [61,62]. The similarity in the characteristics of the SAV1430 and Src SH2 ligand binding sites, and the fact that SAV1430 binds pTyr, further supports the general proposal that SAV1430 functions by forming a protein-protein complex.

Rosetta Stone [63] analysis suggests hypothetical protein SAV0936 may be a binding partner of SAV1430. SAV0936 exhibits 47% sequence identity with the N-terminal region of the C-terminal NifU domain. NifU is a multi-protein complex that is a critical component of the [Fe-S] cluster assembly pathway [64-66] and is essential for the viability of bacteria [67]. A more exhaustive sequence analysis of SAV1430, based on the results with SAV0936, indicates the protein shares ~30% sequence identity with the C-terminal region of the C-terminal domain of the NifU multi-domain structure. These results imply that SAV1430 may interact with SAV0936 to form a complex that exhibits similar activity as the full length NifU domain or may regulate NifU activity. Thus, inhibiting the SAV1430-SAV0936 complex formation may represent a novel target for developing next generation antibiotics.

Conclusion

FAST-NMR provides a high-throughput approach to obtain functional assignments for hypothetical proteins, based on experimentally-determined protein-ligand interactions. FAST-NMR also addresses the current lack in high-throughput experimental methods to obtain functional information [68] where current methods [14] primarily rely on sequence similarity to confer function despite error rates as high as 30% [15,16]. FAST-NMR is based on basic tenets of biochemistry where detailed structural information of a protein-ligand interaction is paramount for understanding the function of a protein. Active-site residues are evolutionarily stable relative to the remainder of the protein's sequence decreasing the likelihood of annotation errors [17,18]. FAST-NMR compliments the success of the Human Genome Project and the Protein Structure Initiative by providing a means to annotate functionally the rapidly expanding number (~2,200) of hypothetical proteins currently deposited in the PDB [31]. Understanding protein function is a paramount necessity for drug discovery programs ability to make successful contributions to human health issues.

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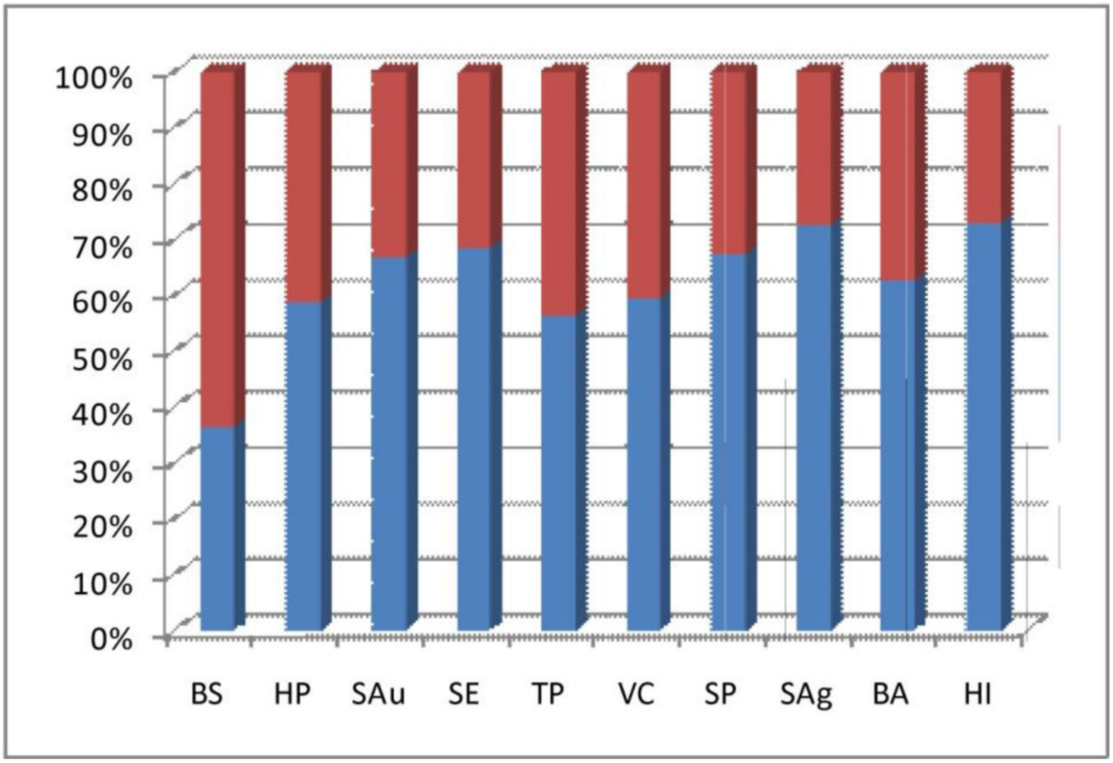


Figure 1.

Functional analysis of bacterial genomes. The blue partitions are the percentage of proteins that have assigned functional categories; the red partitions are the percentage of unannotated proteins. *Bacillus subtlus* (BS); *Heliobactor pylori* (HP); *Staphylococcus aureus* (SAu); *Staphylococcus epidermis* (SE); Treponema pallidum (TP); Vibrio cholerae (VC); Streptococcus pneumoniae (SP); Streptococcus agalactiae (SAg); Bacillus anthracis (BA); Haemophilus influenzae (HI).

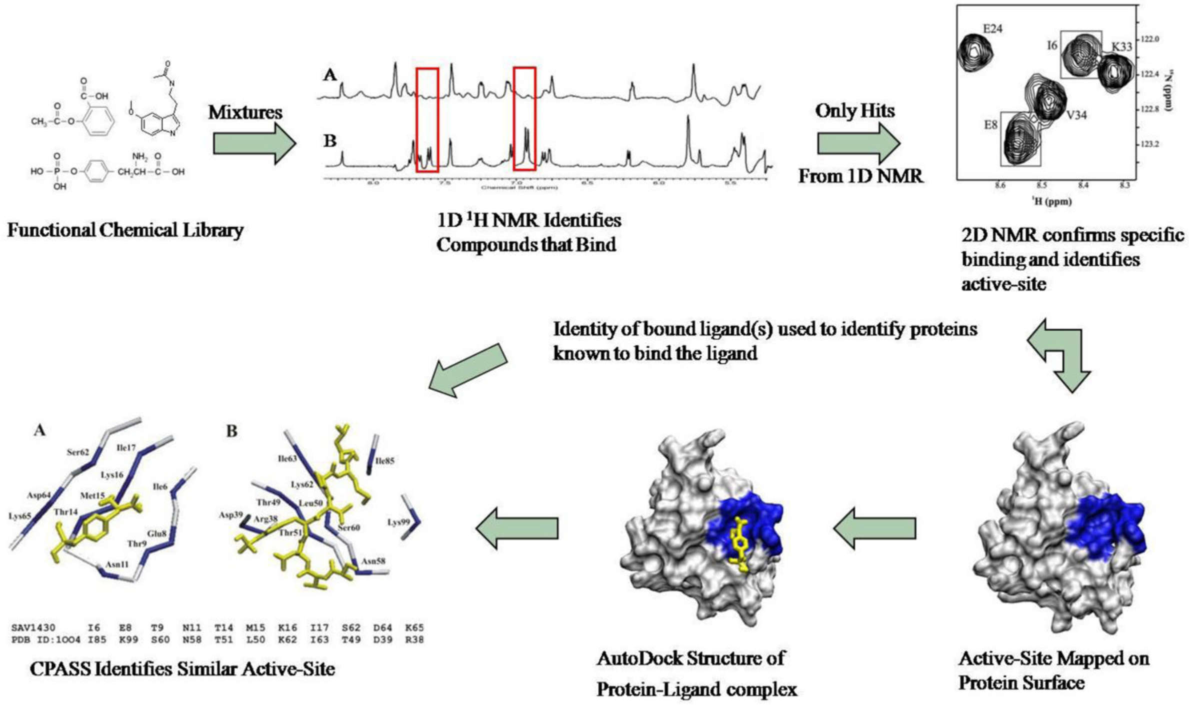

Mixtures
A
B
Functional Chemical Library
1D ^{1}H NMR Identifies
Compounds that Bind
Only Hits
From 1D NMR
E24
I6
K33
E8
V34
^{1}H (ppm)
^{15}N (ppm)
2D NMR confirms specific binding and identifies active-site
Identity of bound ligand(s) used to identify proteins known to bind the ligand
A
Ser62
Ile17
Lys16
Ile6
Asp64
Met15
Lys65
Thr14
Glu8
Thr9
Asn11
B
Ile63
Ile85
Lys62
Thr49
Leu50
Asp39
Arg38
Thr51
Ser60
Lys99
Asn58
SAV1430 I6 E8 T9 N11 T14 M15 K16 I17 S62 D64 K65
PDB ID:1OO4 I85 K99 S60 N58 T51 L50 K62 I63 T49 D39 R38
CPASS Identifies Similar Active-Site
AutoDock Structure of Protein-Ligand complex
Active-Site Mapped on Protein Surface

Figure 2.

Flow chart of FAST-NMR. The hypothetical proteins are screened against mixtures of ligands from the functional chemical library. Reference 1D ^{1}H spectra of the mixtures are compared to those containing protein, where a hit is identified by changes in NMR line-width. Only the ligands identified as binding in the primary screen are further assayed in the secondary 2D ^{1}H-^{15}N experiment. Chemical shift changes confirm a specific interaction and identify the binding site from mapping of the CSPs on the protein's surface. The binding site and CSPs are utilized to determine a rapid co-structure using AutoDock. This co-structure is then used by CPASS to compare the ligand-defined binding site from the hypothetical protein to all other protein-ligand interactions present in the PDB. A general biological function can then be assigned based on an observed similarity to a ligand-defined binding site for a protein of known function.

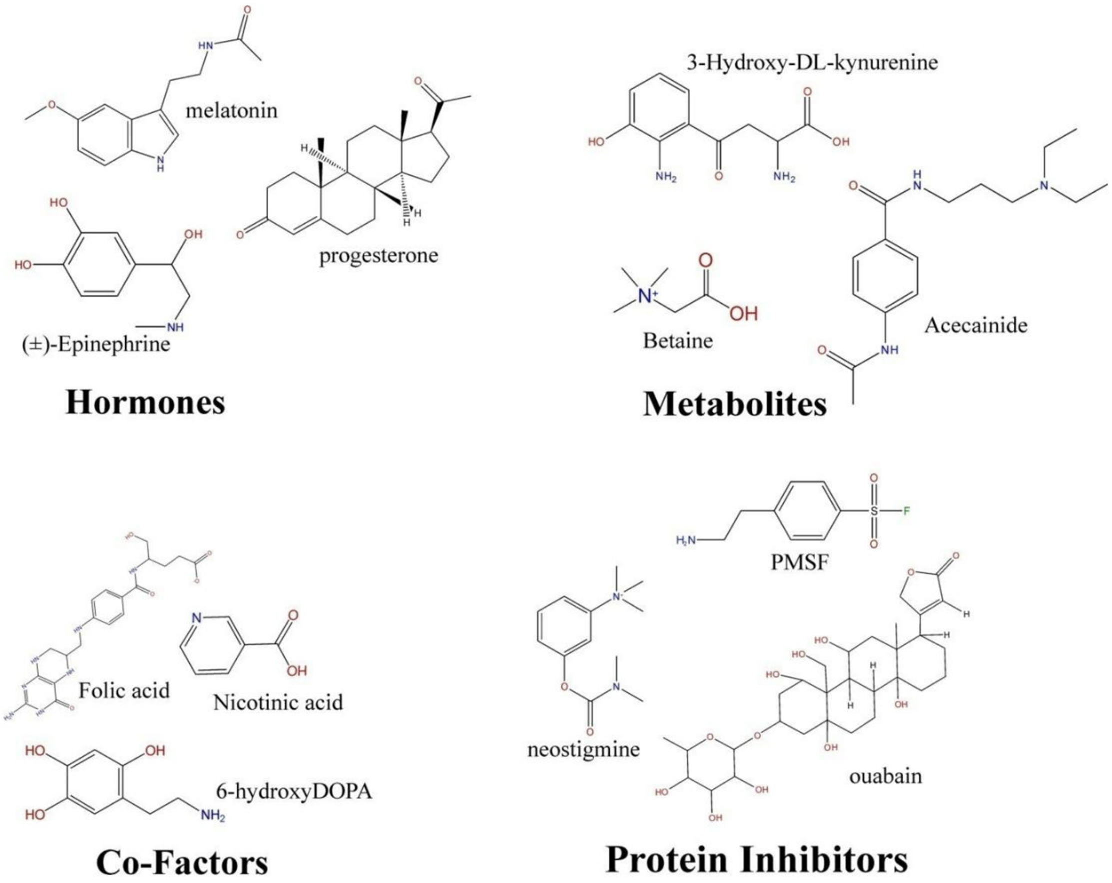
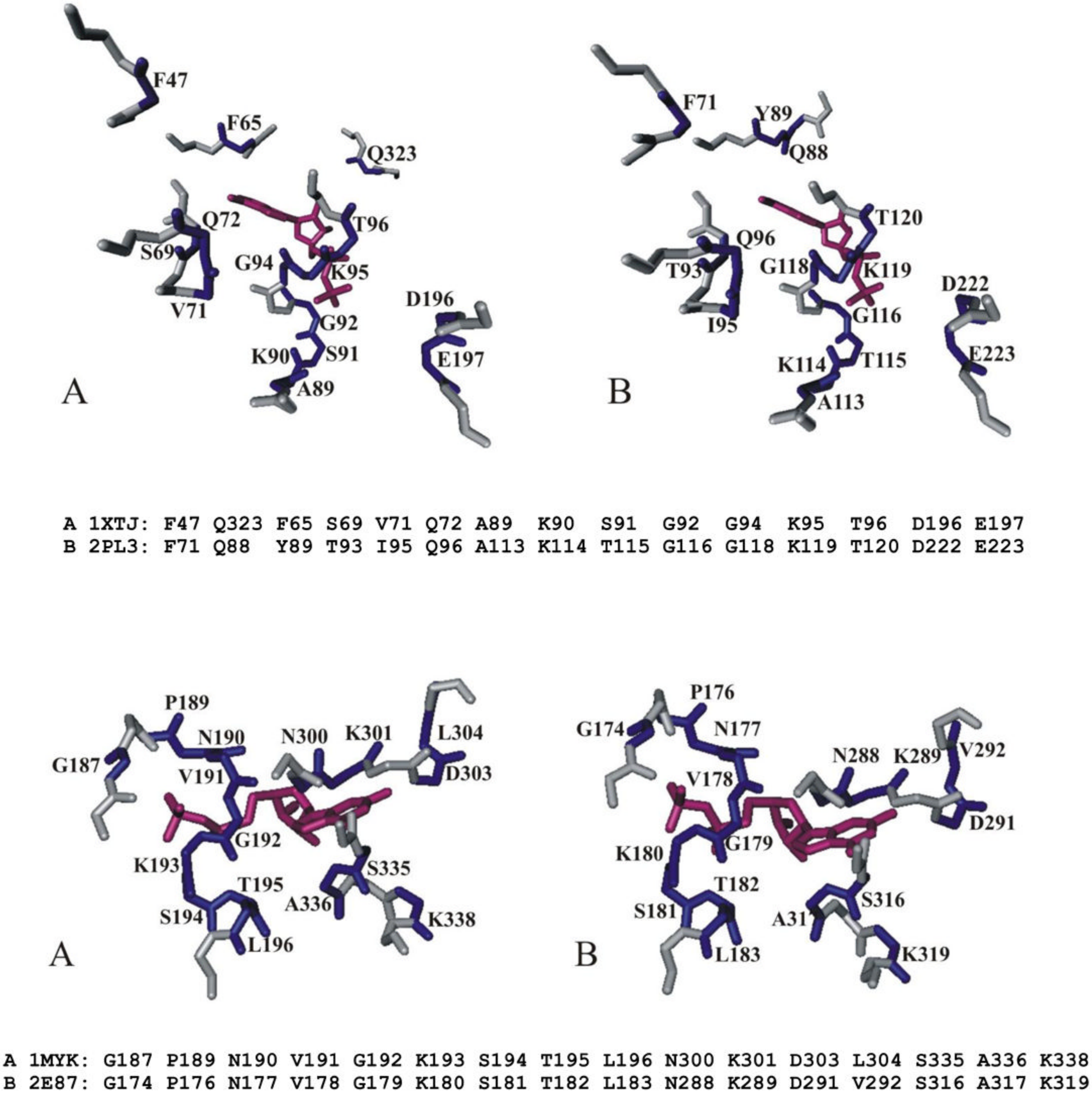


Figure 3.

Functional chemical library. A subset of compounds from four different functional categories from the functional chemical library is displayed. Proteins are screened against mixtures of compounds, and these mixtures were designed to have diverse structure and function to minimize spectral and functional overlap.



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A 1XTJ: F47 Q323 F65 S69 V71 Q72 A89  K90  S91  G92  G94  K95  T96  D196 E197
B 2PL3: F71 Q88  Y89 T93 I95 Q96 A113 K114 T115 G116 G118 K119 T120 D222 E223
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A 1MYK: G187 P189 N190 V191 G192 K193 S194 T195 L196 N300 K301 D303 L304 S335 A336 K338
B 2E87: G174 P176 N177 V178 G179 K180 S181 T182 L183 N288 K289 D291 V292 S316 A317 K319
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Figure 4.

(*top panel*) Binding sites of 1XTJ and 2PL3. Binding-site residues for proteins (A) 1XTJ and (B) 2PL3. Residues within 6 Å of ADP are colored blue and the ligand ADP is colored pink. The amino acid alignment for the ADP binding sites is shown at the bottom of the figure. (*bottom panel*) Binding sites of 1MKY and 2E87. Binding-site residues for proteins (A) 1MKY and (B) 2E87. Residues within 6 Å of GDP are colored blue and the ligand GDP is colored pink. The amino acid alignment for the GDP binding sites is shown at the bottom of the figure.

Table 1

Summary of Applications for Protein Functional Annotation

Method	Advantages/Disadvantages	Website
FAST-NMR [22]	*Advantages* • experimentally identifies ligands that bind protein • experimentally identifies ligand binding site • uses entire description of ligand binding site for functional assignment *Disadvantages* • slower than pure computational methods • requires NMR assignments for protein	http://bionmr-c1.unl.edu
eF-seek [69]	*Advantages* • compares electrostatic surfaces of functional sites to identify ligand binding sites *Disadvantages* • results may identify multiple ambiguous ligand binding sites • protein size limitation • Slow (1-2 days)	http://ef-site.hgc.jp/eF-seek
JAFA [70]	*Advantages* • meta-server to sequence-based methods for functional annotation • does not require a structure *Disadvantages* • redundant with ProcFunc, but lacks structure analysis • sequence similarity, even at the 50% level, is not sufficient to confer function [15]	http://jafa.burnham.org
PDB-UF [27]	*Advantages* • assigns E. C. number to hypothetical proteins in PDB • uses global structural similarity to known enzymes *Disadvantages* • limited to enzymes and accuracy of E. C. assignments • majority of proteins still unassigned	http://pdbuf.bioinfo.pl
ProcFunc [71]	*Advantages* • uses a series of structure-based methods to identify ligand binding sites and potential homologues • comprehensive results from a variety of common methods • fast *Disadvantages* • results may be ambiguous, inconclusive or contradictory • reduced description of ligand binding site, 3-5 amino acids • uncertainty in identifying ligand binding site increases uncertainty in functional annotation	http://www.ebi.ac.uk/thornton-srv/databases/profunc
SuMo [72]	*Advantages* • does not use structure or sequence similarity • accounts for protein flexibility	http://sumo-pbil.ibcp.fr

Method	Advantages/Disadvantages	Website
	Disadvantages • results may identify multiple ambiguous ligand binding sites • uses a reduced description of ligand binding site, searches by triplets of chemical groups • biased to common ligand binding sites in PDB	