

Prediction of function in protein superfamilies

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F1000 Biology Reports 2009, 1:91 (doi:10.3410/BI-91)

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Abstract

Assignment of function for enzymes encoded in sequenced genomes is a challenging task. Predictions of enzyme function can be made using clues from superfamily assignment, structure, genome context, phylogenetic conservation, and virtual screening to identify potential ligands. Ultimately, confident assignment of function requires experimental verification as well as an understanding of the physiological role of an enzyme in the context of the metabolic network.

Introduction and context

Genome sequences are now available for over 900 microbes and 6 multicellular organisms, providing genetic blueprints for organisms that differ enormously in morphology, physiology, and habitat. Unfortunately, our ability to interpret these blueprints is hampered by the lack of assigned function for one-third or more of the proteins in every organism. This commentary will focus specifically on the assignment of enzyme function. Automated assignment of enzyme function is notoriously difficult, as many enzymes with very low sequence identity catalyze the same reaction, and even enzymes that share 98% identity can have different substrate specificities [1].

Efforts to define the roles of enzymes of unknown function often begin with assignment to a superfamily based upon sequence analysis. Enzymes in a superfamily share a common ancestor. In some cases, the ancestral catalytic activity has been retained and divergence has resulted in different substrate specificities. In others, divergence has generated enzymes that catalyze mechanistically distinct reactions, although structural and mechanistic features of the ancestor are conserved.

Superfamily assignment provides clues to enzyme function by indicating the overall fold of the protein, the location of the active site, and the range of known

functions found in superfamily members. Further clues can be provided by conserved sequence motifs. Superfamily members generally share conserved motifs that are important for structure or function or both. Families within a superfamily often have additional motifs and/or patterns of distinct residues within motifs that are involved in substrate specificity or family-specific catalytic functions (Figure 1) [2-6]. Our ability to capitalize on such clues is growing as structural and functional studies expand our knowledge of specific superfamilies. The enolase [3], amidohydrolase [7], and haloalkanoic acid dehalogenase [8] superfamilies are the most thoroughly characterized at this point. However, even in these superfamilies, numerous enzymes fall into families for which there is no known function. Furthermore, some superfamilies do not have easily recognized signatures indicating family membership. The hotdog fold superfamily exhibits little or no conservation of catalytic residues and poorly defined substrate-binding pockets, hindering efforts to use sequence and structural information for the prediction of function [9].

Information about potential functions derived from superfamily affiliation can be exploited along with clues from genome context, phylogenetic conservation, and an understanding of microbial physiology to assign enzyme function [10]. A few of many examples of the use of such information include the identification of

Figure 1. Examples of motifs found in cytochrome maturation proteins and four families of peroxiredoxins

CMP. TlpABc	77-KVILNFWATWCGPCQ-0-QMPDMEAFYKEHK	- 52 -	PTS YFIDTKGVIQDKFIGPMT	-12
CMP. TlpABj	92-KVYLKFWASWCSICL-0-STLADTEDLAKMSD	- 53 -	TEVFIGSDGVLAKKHIGYAK	-12
CMP. cytcmatprotBh	63-GVFLNFWGTYCPPCE-0-RMPHMEKLYGEYK	- 48 -	PTTILINEHGEIVKVTGGMT	-15
CMP. ccbO1	53-RVLINFWTTWCPPCR-0-QMPDMQRFYQDLQ	- 48 -	PTTYMIDSEGIIRHKSYGALT	-12
CMP. cytcbioDr	80-PVVINFWASWCVPCR-0-QAPLFSKLSQETA	- 47 -	PIITFIVDGGQGVVHIKDGPIE	-13
CMP. CcmGBj	81-VSLVNVWASWCVPC-0-DAPLLTELGKDKR	- 46 -	PETFVVGREGTIVYKLVGPIT	-17
Prx1. bfcmprotAp	30-VVLYFYPRAMTPGCT-0-RGVRFNELLDEFE	- 3 -	AVVIGVSTDSVEKNRKFPAEKH	-31
Prx1. PrxTv	31-VVLAFFPGAFSTVCT-0-KMCTFRDSMANFN	- 3 -	AKVIGISVDSPPSLAEFAKKN	-35
Prx1. bfcmprotBh	29-IVLYFYPKDMTPGCT-0-TACDFRDRVEDFK	- 3 -	TVILGVSPDPVERHKKFIEKY	-35
Prx1. bfcmprotCt	30-VLLIFYPGDDTPVCT-0-AQLCDYRNNVAaft	- 3 -	ITVIGISGDSPEshKQFAEKH	-28
Prx1. PrxSc	31-VVLAFFPGAFSTVCT-0-KMCTFRDSMAKFN	- 3 -	AVVIGISVDDPPSNKAFKEQN	-34
Prx2. PrxEc	47-KVLNIFPSIDTGVCA-0-ASVREKQLATEID	- 1 -	TVVLCISADLPFAQSRFCGAB	-34
Prx2. simTPxBs	46-TIISVIPSIDTGVCD-0-AQTRRFNBEAAKLG	- 1 -	VNVYTISADLPFAQARWCGAN	-31
Prx2. hypMm	46-VVLNIFPSIDTPTCA-0-ASVRRFNAEldKLG	- 1 -	VAVL CVSADLPFAHKRF CGAB	-34
Prx2. putTPxBt	46-VLLNIFPSLDTSVCA-0-TSVRKFNKMGAGLK	- 1 -	TVVLAISKDLPFAHARFCTTE	-34
Prx2. simPrxSa	46-KLISVVP SIDTGVCD-0-QQTRKFNSDASKEE	- 0 -	GIVLTI SADLPFAQKRWCASA	-31
Prx2. PrxTpxSp	44-KVLSVVP SIDTGTCS-0-TQTRRFNBEELAGLD	- 1 -	TVVLT V SXDLPFAQKRWC GAB	-31
Prx3. PrxAt	37-VILFGVPGAFPTCS-1-SHVPGFIGKAEELK	- 4 -	DEIICFSVNDP FVMKAWGKTY	-36
Prx3. Prx5Hs	33-GVLPFVPGAFPTGCS-1-THLPGFVQEAEALK	- 4 -	QVVA CLS V NDA PVTGEWGRAH	-38
Prx3. PrxXc	34-VVLFVPGAFPTCS-1-KHLPGYVEQFEAFR	- 3 -	DQVIVVTVDNPFANQAWAKSI	-36
Prx3. PrxSc	48-VIITGAPAAFSPTCT-1-SHLPGYINLDELV	- 5 -	IEVLCTAVNDP FVMQAWGRSQ	-36
Prx3. prxfamprotNo	46-VVLFALPGAFPTVCS-1-NEVPEYIQKLPCLR	- 4 -	DQVA VLA Y NDA VMSAWGKAN	-31
Prx4. PrxEc	26-VVLAFFPLDFTFVCP-0-TINRFSDLKGAFL	- 3 -	AVVLLISCDSVYTHKAWASIP	-36
Prx4. rbcPrxHs	36-VVLFYPLDFTFVCP-0-TIIAFSNRAEDPR	- 3 -	CEVLGVSVD SQFTHLAWINTP	-36
Prx4. PrxTv	32-VVLFYPADFTFVCP-0-TVEGFAEDYEKFK	- 3 -	TEVIVSVD TVVHKAWVQYD	-33
Prx4. ahpcSt	32-SVFFYPADFTFVCP-0-TLDGVADHYEELQ	- 3 -	VDVYSVSTDTHTHKAWHSSS	-33
Prx4. SeCysPrxEa	33-VVLCFYPGDFTFVXA-0-TVSAVAEKYPEFQ	- 3 -	VEVLSVSVDSV FVHKMWNDNB	-36
Prx4. Horf6Hs	33-GILFSPRDFTFVCT-0-TLGRAAKLAPEFA	- 3 -	VKLIALSIDSVEDHLAWSKDI	-45
			RVVVFVGGPKKLLSILYPAT	-73

These proteins are members of the thioredoxin-fold superfamily. Three of the four motifs shown are common to all of the proteins, although specific residues within each motif are diagnostic for specific families. See [4] for identification of sequences. Adapted from [4]. Copyright 2004, American Chemical Society.

function for *o*-succinylbenzoate synthase from *Amycolaptosis* sp. [11], 2,6-dichlorohydroquinone dioxygenase from *Sphingobium chlorophenicum* [12], *N*-formimino-L-glutamate deiminase from *Pseudomonas aeruginosa* [13], and *D*-galacturonate isomerase from *Bacillus halodurans* [14]. However, in many cases, these clues are not enough. For example, protein Cg10062 from *Corynebacterium glutamicum* belongs to the tautomerase superfamily. The protein has six active site residues that are conserved in the superfamily and catalyzes three reactions typical of the superfamily at low rates, but its physiological role still cannot be identified [15].

Additional clues to enzyme function can be obtained by screening libraries of potential substrates for activity (e.g., [16-18]). An example is the identification of function of *Bacillus subtilis* BC0371 [19], which belongs to the muconate-lactonizing enzyme subgroup of the enolase superfamily. This enzyme clusters with the L-Ala-D/L-Glu epimerase family, but three residues typical of that family are missing, suggesting that BC0371 has a different function. The enzyme was incubated with a library of L,L-dipeptides, and epimerization was detected by incorporation of deuterium from the solvent into the substrate. Subsequent kinetic analysis using molecules that were substrates showed that values for k_{cat}/K_M were suspiciously low – at best $10^3/M^{-1}s^{-1}$. Since *N*-acyl amino acid racemases are also found in the muconate-lactonizing enzyme subgroup, a second screening was carried out with a

library of *N*-succinyl L-amino acids. *N*-succinyl-L-Arg was found to be the best substrate; k_{cat}/K_M was $1.4 \times 10^5/M^{-1}s^{-1}$, which is well in the range of values seen for physiologically relevant reactions.

Major recent advances

Virtual screening has been widely used to identify potential enzyme inhibitors for drug discovery efforts by docking a set of ligands into an active site and predicting binding energies based upon van der Waals and electrostatic interactions and solvation effects. This approach has been adapted in recent years to predict substrates for enzymes of unknown function. The true substrates for enzymes are usually found among the high-scoring hits, though often not at the top of the list [20,21]. The correct substrates for 11 enolase superfamily members were found in the top 1% of 19,000 ligands [18]. The substrates for *Pseudomonas putida* mandelate racemase ranked 77 and 140 for docking to a structure of the enzyme from an enzyme-inhibitor complex. Pinpointing the correct substrate is difficult because docking algorithms predict binding affinity, but not propensity for turnover, which requires correct positioning of the substrate with respect to catalytic groups. Furthermore, approximations are required for the scoring function. Finally, it can be difficult to account for conformational changes in the protein that must occur for ligand binding. The docking algorithm can be adapted to allow some flexibility in the protein, but this is not

successful for large conformational changes. Thus, the primary value of virtual screening is in providing clues to structural characteristics of the substrate and thereby limiting the number of potential substrates that must be screened experimentally.

The results of virtual screening are often improved by use of a database containing high-energy intermediates, rather than ground-state substrates, because enzymes generally bind more tightly to transition states or high-energy intermediates than to substrates. To explore this approach, Hermann *et al.* [22] generated 21,000 high-energy forms of 3770 potential substrates for amidohydrolase superfamily enzymes. The docking procedure evaluated up to 1 million poses for each molecule. High-energy intermediates corresponding to the known substrates for seven enzymes were found in the top 100 molecules. For five of the seven enzymes, screening using the ground-state metabolite dataset was considerably less successful. A subsequent study used this approach to predict the function of *Thermatoga maritima* Tm0936, which belongs to the amidohydrolase superfamily [23]. High-energy intermediates based on 4207 potential substrates for amidohydrolase superfamily members were docked into the active site. Nine of the top ten hits were derivatives of adenine, strongly suggesting that the true substrate is some sort of adenine derivative. Only four adenine derivatives were tested experimentally. Two of these (5-methylthioadenosine and 5-adenosylhomocysteine, which were ranked 5 and 6, respectively) were efficiently deaminated, with values of k_{cat}/K_M of greater than $10^5/\text{M}^{-1}\text{s}^{-1}$.

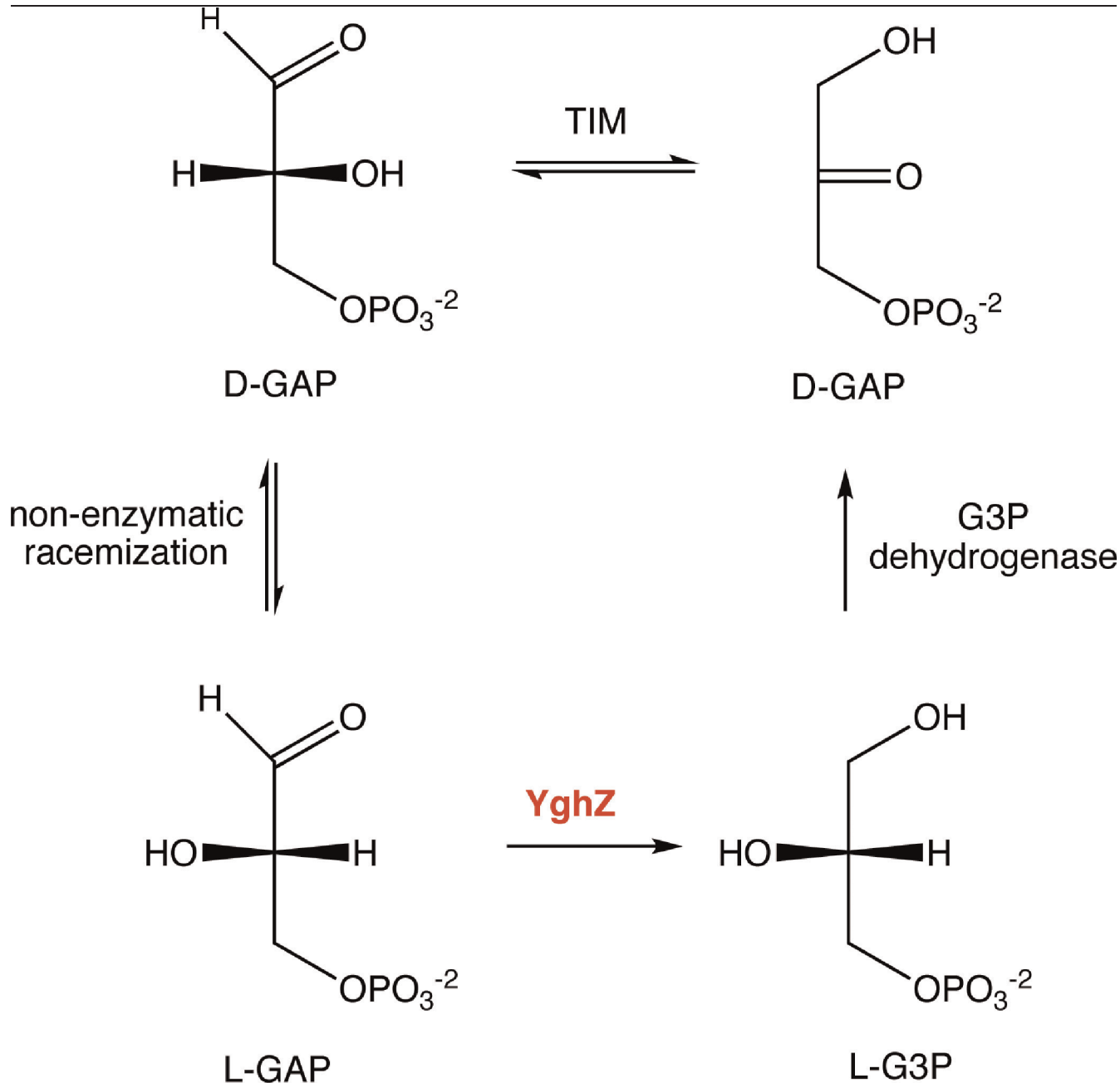
A homology model can be used for virtual screening, although the docking algorithm should be modified to allow subtle rearrangements of side chains in the active site that may not be correctly predicted by the homology model. Song *et al.* [19] generated a homology model for the *Bacillus cereus* protein BC0371, which had been discovered by experimental screening of potential substrates to be an *N*-succinyl-L-Arg racemase (discussed in the 'Introduction and context' section). When a set of 420 L, L-dipeptides and *N*-succinyl L-amino acids was docked, *N*-succinyl-L-Arg ranked 147 when the homology model was used but was the best hit when a flexible-receptor docking protocol was used. Thus, the flexible-receptor protocol allows the virtual screening approach to be extended to enzymes for which a structure is not available. A recent implementation of this strategy led to the prediction and experimental verification that *T. maritima* TM0006 is a dipeptide isomerase with an unusual substrate specificity, preferring dipeptides containing Phe, Tyr, or His in the C-terminal position, in contrast to the structurally characterized L-Ala-D/L-Glu epimerase from *B. subtilis* used as the template for the TM0006 homology model [24].

Future directions

Identifying a substrate that is turned over efficiently *in vitro* is an important step toward understanding the function of an enzyme. Taking this understanding to the next level requires fitting an observed activity into the metabolism of the organism. This is straightforward when an enzyme serves a function in a core catabolic or anabolic pathway, particularly when deletion of the gene encoding the enzyme causes a noticeable phenotype. However, many enzymes for which functions are not known will not play such obvious roles. Some may be involved in unusual metabolic pathways that have not yet been recognized. Others may serve more subtle functions whose discovery requires substantial imagination and understanding of chemistry and metabolism. An interesting example is *Escherichia coli* YghZ, a member of the aldo-ketose reductase superfamily [25]. Overexpression of Ygh allows a strain lacking triose phosphate isomerase (TIM) to grow on lactose, although YghZ does not have TIM activity. A possible function suggested by the superfamily assignment and metabolic context was that YghZ might reduce L-glyceraldehyde 3-phosphate (L-GAP) produced by racemization of D-glyceraldehyde 3-phosphate (D-GAP) (Figure 2). Indeed, YghZ reduces L-GAP with a $k_{\text{cat}}/K_{M,L\text{-GAP}}$ of $4.2 \times 10^5/\text{M}^{-1}\text{s}^{-1}$. The product, L-glycerol 3-phosphate, can be converted to dihydroxyacetone phosphate by glycerol 3-phosphate dehydrogenase. When TIM is present, YghZ may serve to detoxify L-GAP produced by non-enzymatic racemization of D-GAP.

A particular challenge is understanding the physiological roles of enzymes that have broad substrate specificity. For example, *Haemophilus influenzae* YciA hydrolyzes a wide range of CoA thioesters with high efficiency (values of k_{cat}/K_M for 14 substrates are greater than $10^6/\text{M}^{-1}\text{s}^{-1}$) [8]. Similarly, *E. coli* NagD dephosphorylates UMP, GMP, CMP, AMP, and ribose 5'-phosphate with values of k_{cat}/K_M from 2×10^3 to $3 \times 10^4/\text{M}^{-1}\text{s}^{-1}$. The relatively robust activities of these enzymes with multiple substrates suggest that broad specificity may be part of their function. Understanding the physiological roles of such broad-specificity enzymes will require a more sophisticated understanding of the conditions under which they are expressed and the dynamics and relative concentrations of potential substrates that occur as the metabolic network changes in response to environmental conditions.

Identification of function for enzymes of unknown function has necessarily been an endeavor focused upon individual enzymes, and this will continue to be the case since experimental verification is critical for correct annotation of function. However, genomic

Figure 2. Potential role for *Escherichia coli* YghZ

D-GAP, D-glyceraldehyde 3-phosphate; G3P, glycerol 3-phosphate; L-GAP, L-glyceraldehyde 3-phosphate; TIM, triose phosphate isomerase.

enzymologists should take advantage of clues emerging from new high-throughput proteomic methods. Techniques for immobilizing small molecules on microarrays will allow the identification of proteins in complex mixtures that bind to or transform specific molecules or both [26]. Activity-based protein profiling methods identify proteins that react with affinity probes designed

to modify active sites of specific classes of enzymes [27]. Some of the hits obtained in such experiments will undoubtedly be enzymes of unknown function. Such information provides an additional source of clues for identification of function for enzymes for which superfamily analysis, genome context, and phylogenetic analysis have not been sufficient.

Abbreviations

AMP, adenosine monophosphate; CMP, cytidine monophosphate; D-GAP, D-glyceraldehyde 3-phosphate; GMP, guanosine monophosphate; L-GAP, L-glyceraldehyde 3-phosphate; TIM, triose phosphate isomerase; UMP, uridine monophosphate.

Competing interests

The author declares that she has no competing interests.

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