Target selection and annotation for the structural genomics of the amidohydrolase and enolase superfamilies

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Abstract To study the substrate specificity of enzymes, we use the amidohydrolase and enolase superfamilies as model systems; members of these superfamilies share a common TIM barrel fold and catalyze a wide range of chemical reactions. Here, we describe a collaboration between the Enzyme Specificity Consortium (ENSPEC) and the New York SGX Research Center for Structural Genomics (NYSGXRC) that aims to maximize the structural coverage of the amidohydrolase and enolase superfamilies. Using sequence- and structure-based protein comparisons, we first selected 535 target proteins from a variety of genomes for high-throughput structure determination by X-ray crystallography; 63 of these targets were not previously annotated as superfamily members. To date, 20 unique amidohydrolase and 41 unique enolase structures have been determined, increasing the fraction of sequences in the two superfamilies that can be modeled based on at least 30% sequence identity from 45% to 73%. We present case studies of proteins related to uronate isomerase (an amidohydrolase superfamily member) and mandelate racemase (an enolase superfamily member), to illustrate how this structure-focused approach can be used to generate hypotheses about sequence–structure–function relationships.

Keywords Amidohydrolase and enolase superfamilies · Structural genomics · Structure annotation · Target selection

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Abbreviations

PDB: Protein Data Bank
NYSGXRC: New York SGX Research Center for Structural Genomics
ENSPEC: Enzyme specificity consortium
SFLD: Structure function linkage database
PSI: Protein structure initiative
NR: Non-redundant database of protein sequences
ESI: Electrospray ionization
HMM: Hidden Markov Model

Introduction

A long-standing challenge in biology is to predict the molecular function of proteins from their sequences and/or structures. This task is facilitated by a limited number of domain folds [1], restricting the set of structural types that must be studied in deducing a much larger set of functions. Special challenges, however, exist for functional prediction in different classes of proteins. For example, the function of an enzyme often cannot be correctly predicted because there are no clear links from the domain fold to the catalytic function and substrate specificity. Off-setting these problems, studies of genomes and sets of homologous proteins demonstrate that some aspects of catalysis are often conserved between evolutionarily-related proteins, even when these proteins catalyze different overall reactions [2–4]. This empirical observation restricts the functional space that must be considered, further facilitating prediction and leading to definitions of homologous sets of enzymes in terms of protein superfamilies and families based not only on structural conservation, but also on functional conservation [5]: Superfamily members share a common ancestor and potentially some aspects of function, while members of the same family are isofunctional, catalyzing the same overall reaction(s).

The large and diverse amidohydrolase and enolase superfamilies provide a particularly attractive opportunity to study the problem of predicting substrate specificity and enzymatic mechanisms from evolutionary and physical perspectives. These superfamilies are attractive targets because significant knowledge about the specificity of many of their members already exists, while there are still large areas of their sequence space where we do not have any structural or functional information.

Members of the amidohydrolase superfamily catalyze the hydrolysis of a wide range of substrates bearing amide or ester functional groups at carbon and phosphorus centers [6, 7]. A common feature for this superfamily is a mononuclear or binuclear metal center coordinated in a (β/α)8-barrel (TIM barrel) polypeptide chain fold. The active site is formed by loops at the C-terminal ends of the β-strands. Currently, 36 named families have been identified based on the experimentally verified catalytic reactions. The set of superfamily sequences has been clustered into 90 subgroups based on sequence and in some cases active site similarities (the Structure-Function Linkage Database [8]: http://sfld.rbvi.ucsf.edu). In some subgroups, additional information about chemical reactions catalyzed by subgroup members is available; for many of the subgroups, however, no information about functional specificity is available.

Enolase superfamily members catalyze the abstraction of a proton α to a carboxylic acid to form an enolate anion intermediate [9, 10]. Members of this superfamily share an N-terminal α+β capping domain, as well as a C-terminal (β/α)7-barrel domain (modified TIM barrel). The active site is formed by loops at the C-terminal ends of the TIM barrel β-strands and two flexible loops from the capping domain; the active site also includes a Mg2+ ion [11]. Reactions catalyzed by enolases are less diverse than those of the amidohydrolases. The enolases are currently organized into 16 named families and 6 subgroups [8]. Approximately 50% of the sequences in the superfamily are of unknown function.

The amidohydrolase and enolase superfamilies are the focus of our Enzyme Specificity Consortium (ENSPEC), members of which include protein crystallographers, enzymologists, and computational biologists. We aim to predict the substrate specificity of an enzyme based on its experimentally determined and/or modeled structure.
This goal has been enabled by determination of crystallographic structures representing many of the amidohydrolase and enolase families.

To maximize the number of experimentally determined structures, ENSPEC has collaborated with the New York SGX Research Center for Structural Genomics (NYSGXRC), which is one of the four large-scale production centers of the Protein Structure Initiative (PSI) (http://www.nigms.nih.gov/Initiatives/PSI; [43]). NIH guidelines mandate that 70% of the PSI targets come from diverse protein families selected by and shared among the four production centers [43]. About 15% of the targets are reserved for proteins of biomedical relevance defined by each center, and the remaining 15% are “community-nominated” targets. Several hundred of the NYSGXRC community targets are amidohydrolases and enolases nominated by ENSPEC. To date, the collaboration has determined 25 amidohydrolase and 50 enolase structures, contributing substantially to the total of 154 amidohydrolase and 89 enolase structures in the Protein Data Bank (PDB; 6/16/08) [44].

We begin by outlining the data sources and methods used for target selection and structure-based functional annotation (Materials and Methods). Second, we present the results of the target selection process, the status of the selected targets in the structural genomics pipeline, and the improvement in the modeling of the amidohydrolase and enolase superfamilies made possible by the new crystallographic structures (Results and Discussion). We conclude by discussing the biological impact of two sample target structures.

Materials and methods

Target selection

Target selection begins by identifying sequences of known members of the superfamilies (seed sequences), followed by filtering to obtain an initial target list. To identify additional members, we applied sequence- and structure-based expansion methods, followed by filtering for source organisms preferred by NYSGXRC. Superfamily membership for the additional targets was verified by expert curators by inspecting their sequences for probable catalytic residues. A web-based target selection tool was also constructed for further manual filtering to obtain the final target list.

Seed sequence sources

Verified amidohydrolase and enolase superfamily sequences (i.e., seed sequences) were obtained from the Structure Function Linkage Database (SFLD; http://sfld.rbvi.ucsf.edu/) [8]. The SFLD database is a manually constructed database that classifies enzymes hierarchically, based on specific sequence, structure, and functional criteria. The database is updated by a semi-automated method that detects new superfamily members by matching their sequences to Hidden Markov Models trained using the sequences of verified superfamily members, with subsequent manual inspection to verify the presence of catalytic residues. In June 2005, when our target list was constructed, the SFLD contained 3,701 amidohydrolases and 1,795 enolases,1

Filtering of seed sequences

PSI guidelines require that structural genomics targets share ~30% or less amino acid sequence identity to a known three-dimensional structure. To satisfy this condition, the seed amidohydrolase and enolase sequences were processed using the automated comparative modeling server MODWEB (http://salilab.org/modweb) [45]. Sequences with more than 30% sequence identity to any structure in the PDB over at least 70% of their length were identified and excluded from further consideration.

Sequence-based expansion of amidohydrolase and enolase superfamily members

For each seed amidohydrolase and enolase, homologous sequences in the UNIPROT database [46] were identified by the BUILD_PROFILE routine of MODELLER-9 [45]. BUILD_PROFILE is an iterative database-searching tool that relies on local dynamic programming to generate alignments and a robust estimate of their statistical significance. This method identified additional potential amidohydrolase and enolase sequences that were not present in the seed sequence pools.

Structure-based expansion of amidohydrolase superfamily members

In addition to the SFLD entries, we also used the known amidohydrolase superfamily structures to find additional potential amidohydrolase superfamily members (this expansion was not performed for the enolase superfamily). We began by splitting 100 PDB files containing known amidohydrolase structures (June 2005) into separate monomeric structures and clustering them at 80% sequence identity. The resulting 45 non-redundant structures were

1 The numbers of sequences in the publicly accessible version of the SFLD differ from those cited here because large numbers of sequences are undergoing curation at any given time and are therefore not yet listed on the public site.
used for comparative modeling using the automated modeling server MODWEB [45].

First, each structure sequence was used as a query to find its homologs in UNIPROT using PSI-BLAST [47]. Second, these homologs were modeled using the corresponding structure as a template. All models were deposited in our comprehensive MODBASE database of comparative protein structure models (http://salilab.org/modbase/; direct links to the datasets can be found in the supplemental materials) [48]. In addition, the amidohydrolase homologs found in UNIPROT were filtered by removing known amidohydrolase superfamily members, and then subjected to standard comparative modeling with MODWEB using all non-redundant chains in the PDB as potential templates. This step allowed us to eliminate sequences that are likely members of other superfamilies, judged by sequence identity and coverage.

Filtering by organism

While seed sequences could come from any genome, the additional amidohydrolase sequences identified by sequence- and structure-based expansions were filtered for ease of cloning to include only 79 organisms with genomic DNA available to NYSGXRC in 2005 and the marine metagenome from the Sargasso Sea sequencing project (formerly called environmental sequences) [49]. For simplicity, we call the 79 genomes plus the marine metagenome the “NYSGXRC genomes” (Table 1). The NYSGXRC reagent genomes have since been expanded to include over 115 organisms.

Verification of catalytic residues

The putative amidohydrolase sequences resulting from the sequence- and structure-based expansions were aligned to existing amidohydrolase Hidden Markov Models (HMMs) in the SFLD and manually inspected for probable catalytic residues. The final target list only includes sequences with at least 70% of the catalytic residues present.

Target selection tool

For final manual filtering of the target list, we constructed a web-based target selection tool. The tool comprises a combination of MySQL database tables with an interactive web-interface using LAMP [50]. It contains information about the sequences, including UNIPROT annotation, organism, sequence length, closest known structure, sequence identity to other cluster members, and domain boundaries for the TIM barrel domain obtained from SFLD. The interface allows searching for project datasets, organism groups, homologs based on sequence identity, and clusters of related sequences; the resulting sequences can be flagged for rejection or inclusion into the final target list.

Analysis of the target structures

The amidohydrolase and enolase superfamilies were annotated using computational tools. Cytoscape clustering gives an overview of how the targets are distributed across the superfamily [51]. Also, template-based modeling determines how many new sequences can be modeled with the new structural information [45].

Sequence clustering of amidohydrolase superfamily by cytoscape

The time required to perform BLAST searches against the NCBI non-redundant database (NR) of protein sequences [52] was prohibitive due to the size and complexity of the superfamily. Thus, a custom database was created containing only the amidohydrolase sequences in the SFLD. To generate the all-by-all connections for cytoscape clustering, BLAST searches were then performed against this database at an E-value cutoff of $10^{-10}$, using each sequence in the set as a query. Because this custom database contained only sequences known to be members of the amidohydrolase superfamily, the generation of E-values is biased. Consequently, the E-values from this analysis cannot be directly compared to those calculated by BLAST against the NCBI NR database. A cytoscape [51] network was created from these BLAST results. In the absence of established statistical techniques for selecting the E-value cutoff, we examined the superfamily networks at a number of different E-value cutoffs, and present here only one of the corresponding networks, at an E-value cutoff of $10^{-10}$. Further discussion regarding choosing and interpreting E-value cutoffs for sequence similarity networks may be found in [53]. Each node in the network represents a single sequence and each edge represents the pairwise connection between two sequences with the most significant BLAST E-value (better than the cut-off) connecting the two sequences. Lengths of edges are not meaningful, except that sequences in tightly clustered groups are more similar to each other than sequences with few connections. The nodes were arranged using the yFiles organic layout provided in Cytoscape version 2.4. Tools for visualization of protein networks were created by the UCSF Resource for Biocomputing, Visualization, and Informatics (http://www.rbvi.ucsf.edu).

Sequence clustering of enolase superfamily by cytoscape

To generate the all-by-all connections for cytoscape clustering, BLAST analysis was performed against the NR
database, using the sequences in the mandelate racemase-like, glucarate dehydratase-like, mannonate dehydratase-like, and muconate cycloisomerase-like subgroups of the SFLD enolase superfamily. The enolase subgroup was not included in this analysis. Almost all of the enolase subgroup members are in the enolase family, the sequences of which are all iso-functional, i.e. they all perform the well-characterized enolase reaction, important in glycolysis. Only hits in the aforementioned subgroups were used for further analysis. The cytoscape network was created as described above, but using an E-value cutoff for this superfamily of $10^{-40}$.

Table 1 List of 80 NYSGXRC genomes (as of June 2005)

| Organism                  | Taxonomy ID     | Organism                           | Taxonomy ID     |
|---------------------------|-----------------|------------------------------------|-----------------|
| Aeropyrum pernix          | 56636           | Listeria monocytogenes             | 1639            |
| Aquifex aeolicus          | 63363           | Metagenome sequences (Gene synthesis) | 256318         |
| Arabidopsis thaliana      | 3702            | Methanococcus jannaschi            | 2190            |
| Archaeoglobus fulgidus    | 2234            | Mus musculus                       | 10090           |
| Bacillus cereus           | 1396            | Mycobacterium tuberculosis H37Rv   | 83332           |
| Bacillus halodurnas       | 86665           | Mycoplasma pneumonia                | 2104            |
| Bacillus subtilis         | 1423            | Neisseria gonorrhoeae              | 485             |
| Bacillus thurongiensis    | 1428            | Neisseria meningitidis             | 487             |
| Bartonella henselae       | 38323           | Nostoc                             | 1180            |
| Bordetella pertussis      | 520             | Oryctolagus cuniculus              | 9986            |
| Borrelia burgdorferi      | 139             | Oryza sativa                       | 4530            |
| Bos taurus                | 9913            | Ovis aries                         | 9940            |
| Caenorhabditis elegans    | 6239            | Porphyromonas gingivalis           | 837             |
| Campylobacter jejuni      | 197             | Pseudomonas aeruginosa             | 287             |
| Candida albicans          | 5476            | Pseudomonas putida                 | 303             |
| Canis familiaris          | 9615            | Pyrococcus furiosus                | 2261            |
| Capra hircus              | 9925            | Pyrococcus horikoshii              | 53953           |
| Caulobacter vibrioides    | 155892          | Rattus norvegicus                  | 10116           |
| Clostridium acetobutylicum| 1488            | Rhodobacter sphaeroides            | 1063            |
| Corynebacterium diphtheriae| 1717            | Saccharomyces cerevisiae           | 4932            |
| Cryptococcus neoformans   | 5207            | Salmonella typhimurium             | 602             |
| Cryptosporidium parvum    | 5807            | Schizosaccharomyces pombe          | 4896            |
| Deinococcus radiodurans   | 1299            | Shigella Flexneri type 2a          | 42897           |
| Desulfovibrio vulgaris    | 881             | Simian immunodeficiency virus      | 11723           |
| Dicyostelium discoideum   | 44689           | Staphylococcus aureus              | 1280            |
| Drosophila melanogaster   | 7227            | Staphylococcus epidermidis         | 1282            |
| Enterobacter cloacae      | 550             | Streptococcus mutans               | 1309            |
| Enterococcus faecalis     | 1351            | Streptococcus pneumoniae           | 1313            |
| Equus caballus            | 9796            | Streptococcus pyogenes             | 1314            |
| Escherichia coli          | 562             | Sulfolobus solfataricus            | 2287            |
| Escherichia coli 0157:H7  | 83334           | Sus scrofa                         | 9823            |
| Felis catus               | 9685            | Takifugu rubripes                  | 31033           |
| Gallus gallus             | 9031            | Thermoplasma acidophilum           | 2303            |
| Haemophilus influenzae    | 727             | Thermoplasma volcanium             | 50339           |
| Halobacterium sp. NRC-1   | 64091           | Thermotoga maritima                | 2336            |
| Helicobacter pylori       | 210             | Ureaplasma urealyticum             | 2130            |
| Homo sapiens              | 9606            | Vibrio cholerae                    | 666             |
| Human immunodeficiency virus type 1   | 11676          | Xenopus laevis                     | 8355            |
| Klebsiella pneumoniae     | 573             | Xylella fastidiosa                 | 2371            |
| Legionella pneumophila    | 446             | Zea mays                           | 4577            |
Table 2 Summary of new enolase and amidohydrolase X-ray crystal structures and automated template-based modeling results, including subgroup and family assignments

| PDB code | Database accession number (GenPept GI IDs) | No of sequences in Psi-blast alignment | No of sequences with acceptable models and/or fold assignments | No of models >50% seq. ID (min 50% template coverage) | No of models 30-50% seq. ID (min 50% template coverage) | No of models <30% seq. ID | Subgroup assignment | Family assignment |
|----------|--------------------------------------------|---------------------------------------|---------------------------------------------------------------|------------------------------------------------------|------------------------------------------------------|--------------------------|------------------|-------------------|
| 2GL5     | 16420812                                   | 2,863                                 | 2,777                                                         | 0                                                    | 98                                                   | 2,462                    | Mandelate racemase-like | Galactonate dehydratase |
| 2GDQ     | 2633433                                    | 2,234                                 | 2,129                                                         | 1                                                    | 0                                                    | 2,036                    | Mandelate racemase-like | None |
| 2GSH     | 16420830                                   | 2,588                                 | 2,286                                                         | 16                                                   | 9                                                    | 2,110                    | Mandelate racemase-like | L-Fuconate dehydratase |
| 2HNE     | 21115341                                   | 2,746                                 | 2,712                                                         | 83                                                   | 20                                                   | 2,527                    | Mandelate racemase-like | None |
| 2HZG     | 77386310                                   | 2,667                                 | 2,341                                                         | 1                                                    | 1                                                    | 2,248                    | Mandelate racemase-like | None |
| 2ISQ     | 15832389                                   | 2,566                                 | 2,340                                                         | 21                                                   | 13                                                   | 2,206                    | Mandelate racemase-like | None |
| 2NQL     | 17743914                                   | 2,849                                 | 2,470                                                         | 2                                                    | 1                                                    | 2,356                    | Mandelate racemase-like | None |
| 2O56     | 16767118                                   | 3,016                                 | 2,968                                                         | 15                                                   | 127                                                  | 2,735                    | Mandelate racemase-like | None |
| 2OQH     | 21225834                                   | 2,690                                 | 2,668                                                         | 2                                                    | 32                                                   | 2,630                    | Glucarate dehydratase-like | None |
| 2OQY     | 23100298                                   | 2,700                                 | 2,631                                                         | 1                                                    | 0                                                    | 3,004                    | Muconate cycloisomerase-like | None |
| 2OVL     | 21221904                                   | 2,670                                 | 2,656                                                         | 1                                                    | 97                                                   | 2,534                    | Mandelate racemase-like | None |
| 2OG9     | 91786345                                   | 2,669                                 | 2,664                                                         | 10                                                   | 75                                                   | 2,553                    | Mandelate racemase-like | L-Talarate/galactarate dehydratase |
| 2OLA     | 88195610                                   | 2,719                                 | 2,697                                                         | 5                                                    | 3                                                    | 2,652                    | Muconate cycloisomerase-like | o-Succinylbenzoate synthase |
| 2O06     | 91778214                                   | 3,271                                 | 3,221                                                         | 3                                                    | 2                                                    | 3,111                    | Mandelate racemase-like | None |
| 2OKT     | 57650581                                   | 2,723                                 | 2,705                                                         | 5                                                    | 3                                                    | 2,664                    | Muconate cycloisomerase-like | o-Succinylbenzoate synthase |
| 2OPJ     | 72161814                                   | 2,562                                 | 1,855                                                         | 19                                                   | 31                                                   | 1,712                    | Mandelate racemase-like | o-Succinylbenzoate synthase |
| 2OX4     | 56552160                                   | 2,733                                 | 2,639                                                         | 11                                                   | 136                                                  | 2,449                    | Mandelate racemase-like | None |
| 2OZ3     | 67154209                                   | 2,743                                 | 2,656                                                         | 38                                                   | 25                                                   | 2,567                    | Mandelate racemase-like | None |
| 2OZ8     | 13475907                                   | 2,821                                 | 2,674                                                         | 0                                                    | 0                                                    | 2,641                    | Mandelate racemase-like | None |
| 2PO1     | 46136735                                   | 2,747                                 | 2,661                                                         | 13                                                   | 52                                                   | 2,561                    | Mandelate racemase-like | None |
| 2OZT     | 22294898                                   | 2,816                                 | 2,726                                                         | 0                                                    | 16                                                   | 2,722                    | Muconate cycloisomerase-like | o-Succinylbenzoate synthase |
| 2PCE     | 83951697                                   | 2,693                                 | 2,683                                                         | 1                                                    | 16                                                   | 2,635                    | Muconate cycloisomerase-like | None |
| 2PGE     | 51244103                                   | 2,779                                 | 2,767                                                         | 1                                                    | 19                                                   | 2,768                    | Muconate cycloisomerase-like | o-Succinylbenzoate synthase |
| 2PGW     | 16263250                                   | 2,781                                 | 2,743                                                         | 1                                                    | 3                                                    | 2,694                    | Mandelate racemase-like | None |
| 2PMQ     | 114764387                                  | 2,881                                 | 2,760                                                         | 3                                                    | 14                                                   | 2,723                    | Muconate cycloisomerase-like | None |
| 2POD     | 53723090                                   | 2,745                                 | 2,732                                                         | 12                                                   | 97                                                   | 2,585                    | Mandelate racemase-like | Galactonate dehydratase |
| 2POZ     | 13488170                                   | 2,861                                 | 2,836                                                         | 1                                                    | 166                                                  | 2,687                    | Mandelate racemase-like | None |
| 2PPG     | 16262827                                   | 2,947                                 | 2,755                                                         | 2                                                    | 66                                                   | 2,707                    | Mandelate racemase-like | None |
| 2PS2     | 83774494                                   | 2,777                                 | 2,753                                                         | 3                                                    | 16                                                   | 2,712                    | Muconate cycloisomerase-like | None |
| 2QDE     | 56478643                                   | 2,930                                 | 2,670                                                         | 1                                                    | 62                                                   | 2,595                    | Muconate cycloisomerase-like | None |
| 2QGY     | 110347373                                  | 2,988                                 | 2,899                                                         | 0                                                    | 1                                                    | 2,912                    | Mandelate racemase-like | None |
| 2QQ6     | 108803396                                  | 3,238                                 | 3,216                                                         | 0                                                    | 201                                                  | 3,081                    | Mandelate racemase-like | Galactonate dehydratase |
| 2QYE     | 83951695                                   | 3,128                                 | 3,121                                                         | 0                                                    | 21                                                   | 3,110                    | Muconate cycloisomerase-like | None |
Table 2 continued

| PDB code | Database accession number (Genpept GI IDs) | No of sequences in Psi-blast alignment | No of sequences with acceptable models and/or fold assignments | No of models >50% seq. ID (min 50% template coverage) | No of models 30-50% seq. ID (min 50% template coverage) | No of models <30% seq. ID (min 50% template coverage) | Subgroup assignment | Family assignment |
|----------|------------------------------------------|--------------------------------------|---------------------------------------------------------------|-----------------------------------------------------|-----------------------------------------------------|-----------------------------------------------------|-------------------|------------------|
| 3BJS     | 6791043                                  | 3,261                                | 2,897                                                         | 4                                                   | 82                                                  | 2,810                                                              | Mandelate racemase-like | None             |
| 2QDD     | 83951694                                 | 2,868                                | 2,852                                                         | 0                                                   | 20                                                  | 2,849                                                              | Muconate cycloisomerase-like | o-Succinylbenzoate synthase |
| 3CAW     | 42522147                                 | 2,220                                | 2,139                                                         | 0                                                   | 0                                                   | 2,137                                                              | Muconate cycloisomerase-like | o-Succinylbenzoate synthase |
| 3CT2     | 70731221                                 | 3,483                                | 2,771                                                         | 84                                                  | 77                                                  | 2,667                                                              | Muconate cycloisomerase-like | Muconate cycloisomerase |
| 3CYJ     | 108805509                                | 3,551                                | 2,879                                                         | 8                                                   | 35                                                  | 2,838                                                              | Mandelate racemase-like | None             |
| 3DDM     | 33575875                                 | 5,603                                | 3,576                                                         | 6                                                   | 27                                                  | 3,591                                                              | Mandelate racemase-like | None             |
| 3BSM     | 92115090                                 | 3,372                                | 3,359                                                         | 86                                                  | 165                                                 | 3,097                                                              | Mannionate dehydratase-like | Mannionate dehydratase |
| Total (unique sequences) | 7,013                                    | 5,804                                | 398                                                           | 766                                                 | 5,190                                               |                                                                     |                   |                  |

**Amidohydrolase superfamily**

| PDB code | Database accession number (Genpept GI IDs) | No of sequences in Psi-blast alignment | No of sequences with acceptable models and/or fold assignments | No of models >50% seq. ID (min 50% template coverage) | No of models 30-50% seq. ID (min 50% template coverage) | No of models <30% seq. ID (min 50% template coverage) | Subgroup assignment | Family assignment |
|----------|------------------------------------------|--------------------------------------|---------------------------------------------------------------|-----------------------------------------------------|-----------------------------------------------------|-----------------------------------------------------|-------------------|------------------|
| 2GOK     | 17742376                                 | 3,001                                | 2,943                                                         | 96                                                  | 103                                                 | 2,678                                                              | Imidazolonepropinase-like | Imidazolonepropinase |
| 2OOD     | 27378991                                 | 3,609                                | 3,572                                                         | 0                                                   | 160                                                 | 3,440                                                              | Guanine deaminase-like | None             |
| 2OOF     | 83646866                                 | 3,588                                | 3,578                                                         | 142                                                 | 154                                                 | 3,270                                                              | Imidazolonepropinase-like | Imidazolonepropinase |
| 2SG5     | 9951721                                  | 569                                  | 448                                                           | 28                                                  | 50                                                  | 340                                                                | None               | None             |
| 2BU      | 15023121                                 | 3,386                                | 3,263                                                         | 5                                                   | 96                                                  | 3,198                                                              | Newfam59            | None             |
| 2ICS     | 29342885                                 | 3,433                                | 3,334                                                         | 3                                                   | 28                                                  | 3,209                                                              | Unknown18            | None             |
| 2MR      | 9911007                                  | 3,790                                | 3,502                                                         | 1                                                   | 3                                                   | 3,498                                                              | None               | None             |
| 2OGJ     | 17741648                                 | 3,527                                | 3,510                                                         | 5                                                   | 18                                                  | 3,395                                                              | Newfam71            | None             |
| 2PB5     | 23466009                                 | 3,319                                | 3,302                                                         | 2                                                   | 37                                                  | 3,230                                                              | Unknown41           | None             |
| 2PAI     | 91783796                                 | 3,264                                | 3,252                                                         | 4                                                   | 116                                                 | 3,128                                                              | Unknown55            | None             |
| 2PQ1     | 13428263                                 | 460                                  | 263                                                           | 35                                                  | 14                                                  | 172                                                                | Uronate isomerase-like | Uronate isomerase |
| 2PQ6     | 15615056                                 | 306                                  | 189                                                           | 3                                                   | 0                                                   | 167                                                                | Uronate isomerase-like | Uronate isomerase |
| 2QS8     | 114773165                                | 3,508                                | 3,497                                                         | 15                                                  | 144                                                 | 3,280                                                              | Unknown43            | None             |
| 2QT3     | 32455889                                 | 3,723                                | 3,693                                                         | 1                                                   | 49                                                  | 3,606                                                              | Unknown95            | None             |
| 2RAG     | 16126978                                 | 911                                  | 602                                                           | 7                                                   | 53                                                  | 502                                                                | Newfam32            | None             |
| 2R8C     | 4447959                                  | 3,649                                | 3,632                                                         | 19                                                  | 195                                                 | 3,359                                                              | Unknown47            | None             |
| 2BU      | 15023121                                 | 3,386                                | 3,363                                                         | 5                                                   | 96                                                  | 3,198                                                              | Newfam59            | None             |
| 2OOF     | 83646866                                 | 3,588                                | 3,578                                                         | 142                                                 | 154                                                 | 3,270                                                              | Imidazolonepropinase-like | Imidazolonepropinase |
| 3BAO     | 9948434                                  | 1,149                                | 656                                                           | 16                                                  | 34                                                  | 504                                                                | Newfam190           | None             |
| 3CJP     | 15896580                                 | 3,289                                | 3,286                                                         | 1                                                   | 1,467                                               | 1,851                                                               | Newfam63            | None             |
| 3BE7     | 4436882                                 | 3,697                                | 3,198                                                         | 4                                                   | 112                                                 | 3,042                                                              | Unknown42            | None             |
| Total (unique sequences) | 12,101                                    | 11,628                               | 302                                                           | 2,429                                               | 8,912                                               |                                                                     |                   |                  |

Only one entry is shown for structures determined in different crystal forms or ligand binding states. An acceptable model is defined to be based on a significant PSI-BLAST E-value (0.0001) or a favorable GA341 model score (>0.7) [60].
Template-based modeling by MODWEB

Automated comparative modeling of all known protein sequences using the new NYSGXRC crystallographic structures as templates was performed with MODWEB [45]. We relied on the MODWEB option that allows using a protein structure as input and results in models for all of the identifiable sequence homologs of the input structure from the NCBI NR database; these homologs were identified during ten PSI-BLAST iterations of the template sequence against NR (E-value cutoff is 0.0001). The results are available at http://salilab.org/modbase/models_nysgxrc_latest.html (Table 2).

Results and discussion

We first present the results of the target selection procedure. We also describe the current snapshot of the progress of the targets through our structural genomics pipeline (June 2008). We then indicate how the resulting crystallographic structures are distributed across the two superfamilies. Next, we determine the number of protein sequences in the comprehensive sequence databases that are detectably related to these protein structures (i.e., the modeling leverage). Finally, for each of the two superfamilies, we describe an example target with interesting biological features.

Target selection

Given the capacities of ENSPEC and NYSGXRC, the goal was to identify approximately 500 target sequences, approximately evenly distributed between the two superfamilies. These targets were obtained by selecting representatives from previously identified superfamily members as well as by identifying new superfamily members in a select set of genomes (Materials and Methods).

Targets for the amidohydrolase superfamily

From the SFLD, we obtained a list of 3,701 amidohydrolase superfamily members. The first filtering step resulted in 1,918 sequences with less than 30% sequence identity to a known structure and at least 250 amino acid residues in length, originating from 424 organisms. We chose the 30% sequence identity limit, in congruence with NIH PSI guidelines, to concentrate our efforts on protein sequences with limited structural knowledge; sequences related at less than 30% sequence identity to the closest known structure are frequently modeled inaccurately due to errors in the corresponding target-template alignments [54–56].

These 1,918 sequences were further filtered manually using the target selection tool to obtain the reduced set of 224 target sequences. The selected amidohydrolase superfamily members are evenly distributed among the various clades of the superfamily, thus representing the diversity within the superfamily. Preference was given to the NYSGXRC genomes, but other organisms were also considered.

The 224 targets can be divided into 76 clusters with less than 30% sequence identity between any pair of sequences from two different clusters, 126 clusters at 50% sequence identity, and 177 clusters at 80% sequence identity. The amidohydrolase superfamily members all contain the defining conserved TIM barrel domain with some variation in their lengths; all targets are between 224 and 628 amino acid residues long, with 90% of them shorter than 500 residues. The length variation stems mostly from loops that connect the main secondary structure elements of the TIM barrel fold and is consistent with the previously observed size range for TIM barrel domains (150 to 500 residues [57]).

In addition to the known superfamily members, the sequence- and structure-based expansions detected 63 putative amidohydrolase superfamily members that were not initially in the SFLD (Table 3). These new potential targets fall into two categories: (i) divergent sequences that were detected by the sequence-based approach (Fig. 1, blue box) and (ii) divergent sequences that were detected by the structure-based approach (Fig. 1, orange box). Of the 63 putative amidohydrolase superfamily sequences, 50 were subsequently verified using the SFLD update procedure. The presence of probable catalytic residues for the remaining 13 targets was verified manually. Nine of these 13 sequences were detected by both the sequence- and structure-based approaches, and four sequences were only detected by the structure-based approach. Thus, the sequence- and structure-based approaches yielded 13 additional targets that could not be identified as amidohydrolase superfamily members using previously available protocols (corresponding to 21% of the new putative members of the amidohydrolase superfamily).

In summary, the final amidohydrolase target list includes 224 previously identified amidohydrolase superfamily members, as well as the 63 newly identified sequences. The final list includes 287 sequences from 53 organisms that cover 22 (61%) of the named families in the superfamily (Fig. 2).

Targets for the enolase superfamily

We used a simpler selection scheme for the enolase superfamily members, because previous detailed studies have effectively found all of the superfamily members in publicly available sequence and structure databases (data not shown). Of the 1,795 sequences already established as enolase superfamily members, we selected as targets the...
| Database ID (GenPept GI IDs) | Method                  | Organism                              | Length | Annotation available at target selection                                                                 | Verification |
|-----------------------------|-------------------------|---------------------------------------|--------|----------------------------------------------------------------------------------------------------------|--------------|
| 7462218                     | Structure-based         | *Thermotoga maritima*                 | 434    | Conserved hypothetical protein                                                                          | HMM          |
| 7497374                     | Structure-based         | *Caenorhabditis elegans*              | 434    | Hypothetical protein C44B7.10                                                                          |             |
| 7500805                     | Structure-based         | *Caenorhabditis elegans*              | 313    | T21966 hypothetical protein F38E11.3—*Caenorhabditis elegans*                                          |             |
| 9948434                     | Structure-based         | *Pseudomonas aeruginosa* PAO1         | 448    | Probable dipeptidase precursor (*Pseudomonas aeruginosa*)                                              |             |
| 10173106                    | Structure-based         | *Bacillus halodurans*                 | 427    | BH0493                                                                                                |             |
| 10175729                    | Structure-based         | *Bacillus halodurans*                 | 571    | DNA-dependent DNA polymerase beta chain                                                                  |             |
| 13700943                    | Structure-based         | *Staphylococcus aureus*               | 570    | DNA-dependent DNA polymerase beta chain                                                                  |             |
| 14600641                    | Structure-based         | *Aeropyrum pernix*                    | 313    | 313aa long hypothetical microsomal dipeptidase                                                         |             |
| 14601853                    | Template                | *Aeropyrum pernix*                    | 394    | Hypothetical protein (*Aeropyrum pernix*)                                                               |             |
| 14602106                    | Structure-based         | *Aeropyrum pernix*                    | 327    | Hypothetical protein (*Aeropyrum pernix*)                                                               |             |
| 15600589                    | Structure-based         | *Pseudomonas aeruginosa* PAO1         | 325    | D82971 hypothetical protein PA5396 (imported)—*Pseudomonas aeruginosa* (strain PAO1)                    |             |
| 15612748                    | Structure-based         | *Bacillus halodurans*                 | 448    | BH0185                                                                                                |             |
| 15614834                    | Structure-based         | *Bacillus halodurans*                 | 310    | Dipeptidase                                                                                             |             |
| 15791917                    | Structure-based         | *Campylobacter jejuni*                | 265    | Hypothetical protein Cj0556                                                                              |             |
| 15805850                    | Structure-based         | *Deinococcus radiodurans* R1          | 418    | Hydrolase, putative                                                                                      |             |
| 15896580                    | Structure-based         | *Clostridium acetobutylicum*           | 262    | Predicted amidohydrolase (dihydroorotase family)                                                        |             |
| 15898656                    | Structure-based         | *Sulfobatus solfatarius*              | 314    | Microsomal dipeptidase                                                                                   |             |
| 15925570                    | Structure-based         | *Staphylococcus aureus*               | 336    | Conserved hypothetical protein                                                                           |             |
| 16125737                    | Structure-based         | *Caulobacter vibrioides*              | 487    | Uronic isomerase (EC 5.3.1.12) (Glucuronate isomerase)                                                   |             |
| 16126978                    | Structure-based         | *Caulobacter vibrioides*              | 417    | Dipeptidase                                                                                             |             |
| 16127409                    | Structure-based         | *Caulobacter vibrioides*              | 353    | Hypothetical protein                                                                                     |             |
| 16130781                    | Structure-based         | *Escherichia coli* K12                | 464    | Soluble protein involved in cell viability at the beginning of stationary phase; soluble protein involved in cell viability at the beginning of stationary phase, contains urease domain |             |
| 16410647                    | Structure-based         | *Listeria monocytogenes*              | 570    | Imo1231                                                                                                |             |
| 17556402                    | Structure-based         | *Caenorhabditis elegans*              | 352    | Hypothetical protein Y71D11A.3a                                                                          |             |
| 19705473                    | Structure-based         | *Rattus norvegicus*                   | 336    | 2-amino-3-carboxyfuconate-6-semialdehyde decarboxylase                                                   |             |
| 19911227                    | Structure-based         | *Homo sapiens*                        | 336    | 2-amino-3-carboxyfuconate-6-semialdehyde decarboxylase                                                   |             |
| 19911231                    | Structure-based         | *Caenorhabditis elegans*              | 401    | 2-amino-3-carboxyfuconate-6-semialdehyde decarboxylase                                                   |             |
| 24379660                    | Structure-based         | *Streptococcus mutans* UA159          | 267    | conserved hypothetical protein                                                                           |             |
| 33592291                    | Structure-based         | *Bordetella pertussis* Tohama I        | 284    | Putative 2-pyrene-4,6-dicarboxylic acid hydrolase                                                        |             |
| 33593502                    | Structure-based         | *Bordetella pertussis* Tohama I        | 341    | Putative dipeptidase                                                                                     |             |
| 39976001                    | Sequence- and structure-based | *Magnaporthe grisea* 70-15           | 417    | Hypothetical protein                                                                                     |             |
| Database ID (GenPept GI IDs) | Method | Organism | Length | Annotation available at target selection | Verification |
|----------------------------|--------|----------|--------|----------------------------------------|--------------|
| 42527610                   | Structure-based | *Treponema denticola* ATCC 35405 | 371 | Dihydroorotase, putative | HMM          |
| 42631159                   | Structure-based | *Haemophilus influenzae* | 330 | Hypothetical protein | HMM          |
| 51012913                   | Structure-based | *Saccharomyces cerevisiae* | 313 | YMR262W | HMM          |
| 51968376                   | Structure-based | *Arabidopsis thaliana* | 346 | Unnamed protein product | HMM          |
| 51968996                   | Structure-based | *Arabidopsis thaliana* | 346 | Unnamed protein product | HMM          |
| 55980841                   | Structure-based | *Thermus thermophilus* HB8 | 369 | Amidohydrolase family protein | HMM          |
| 60279993                   | STRUCTURE-based | *Pseudomonas aeruginosa* | 403 | PvdM HMM | HMM          |
| 66807941                   | Structure-based | *Dictyostelium discoideum* | 359 | Hypothetical protein | HMM          |
| 66808659                   | Structure-based | *Dictyostelium discoideum* | 322 | Hypothetical protein | HMM          |
| 1065989                    | Sequence-based | *Bacillus subtilis* subsp. *subtilis* str. 1 | 577 | Adenine deaminase | HMM          |
| 15023784                   | Sequence-based | *Clostridium acetobutylicum* | 570 | Adenine deaminase | HMM          |
| 24636152                   | Structure-based | *Caenorhabditis elegans* | 403 | Hypothetical protein C44B7.12 | HMM          |
| 29377069                   | Structure-based | *Enterococcus faecalis* V583 | 444 | Chlorohydrolase family protein | HMM          |
| 40788915                   | Structure-based | *Homo sapiens* | 777 | Q93075_chr3:10265710-10295706_H233R_V272I_L374P PUTATIVE DEOXYRIBONUCLEASE KIAA0218 (EC 3.1.21.-) | HMM          |
| 45446932                   | Sequence- and structure-based | *Drosophila melanogaster* | 774 | CG32626-PA, isoform A | HMM          |
| 56203368                   | Sequence- and structure-based | *Homo sapiens* | 776 | Adenosine monophosphate deaminase 1 (isoform M) | HMM          |
| 56203369                   | Sequence-based | *Homo sapiens* | 780 | OTTHUMP0000059283 | HMM          |
| 57230710                   | Structure-based | *Filobasidiella neoformans* | 469 | Hydrolase, putative | HMM          |
| 63055053                   | Structure-based | *Homo sapiens* | 761 | TatD DNase domain containing 2 | HMM          |
| 68250266                   | Structure-based | *Haemophilus influenzae* | 251 | Conserved putative deoxyribonuclease | HMM          |
| 429129                     | Structure-based | *Saccharomyces cerevisiae* | 797 | YB9Z YEAST HYPOTHETICAL 92.9 KD PROTEIN IN SSH1-APE3 INTERGENIC REGION | Manual       |
| 7293948                    | Sequence-based | *Drosophila melanogaster* | 520 | CG5998-PA | Manual       |
| 11463854                   | Sequence-based | *Drosophila melanogaster* | 561 | Male-specific IDGF | manual       |
| 14602062                   | Structure-based | *Aeropyrum pernix* | 375 | Hypothetical protein [Aeropyrum pernix] | Manual       |
| 15898896                   | Structure-based | *Sulfolobus solfataricus* | 269 | Conserved hypothetical protein | Manual       |
| 16264026                   | Template | *Sinorhizobium meliloti* | 466 | HYPOTHETICAL PROTEIN | Manual       |
| 17646150                   | Sequence- and structure-based | *Drosophila melanogaster* | 506 | Adenosine deaminase-related growth factor C | Manual       |
| 23093239                   | Sequence-based | *Drosophila melanogaster* | 561 | CG32178-PA | Manual       |
| 25009707                   | Sequence-based | *Drosophila melanogaster* | 561 | AT05468p | Manual       |
| 33593596                   | Structure-based | *Bordetella pertussis* Tohama 1 | 523 | Conserved hypothetical protein | Manual       |
| 40744823                   | Structure-based | *Aspergillus nidulans* FGSC A4 | 562 | HYPOTHETICAL protein | Manual       |
| 47678365                   | Sequence-based | *Homo sapiens* | 511 | Cat eye syndrome critical region protein 1 [Homo sapiens] | Manual       |
| 49116836                   | Sequence- and structure-based | *Xenopus laevis* | 510 | Hypothetical protein | Manual       |

Tables listing all amidohydrolase and enolase superfamily targets can be found at [http://salilab.org/projects/enspec/](http://salilab.org/projects/enspec/) (HMM Hidden Markov Model verification)
255 sequences with less than 30% sequence identity to a known structure over at least 250 residues in length, originating from 98 organisms. These targets form 74 clusters at the 30% sequence identity cutoff, 126 clusters at 50% sequence identity, and 196 clusters at 80% sequence identity. The length distribution is 200 to 656 amino acid residues, with 90% of the sequences between 200 and 405 residues in length.

A complete list of the selected amidohydrolase and enolase superfamily targets can be found at http://salilab.org/projects/enspec/.

Structural genomics pipeline attrition

To date, 254 amidohydrolase (88%) and 206 enolase (80%) superfamily members have been attempted using the NYSGXRC/ENSPEC X-ray crystallographic structure determination pipeline. Progress to date and attrition rate at each stage of the pipeline are documented in Table 4 (June 2008). The project has not yet been completed, and a number of targets are still progressing through the pipeline. Also, a few targets in the target list have not yet been entered in the experimental pipeline. Therefore, the final overall success rate should be higher than that presented in

Table 4. Experimental results for all NYSGXRC Community-nominated targets are updated weekly in PepcDB (http://pepcdb.pdb.org/).

Clear trends are observed in the success rates of crystallization and subsequent crystallographic structure determination for the amidohydrolase and enolase superfamily members. While only 38% of the purified targets were members of the enolase superfamily, they comprise 67% of the unique experimental structures. If crystals are obtained for an enolase superfamily member, there is a good chance that its structure will be successfully determined. On the other hand, for at least a quarter of the amidohydrolase proteins, we observed unusually broad peaks in the electrospray ionization (ESI) mass spectra of the intact proteins, indicative of heterogeneity in the preparation. Proteolytic digestion followed by tandem mass spectrometry analysis was carried out on the heterogeneous proteins; multiple sites of oxidation and methylation were identified with 90% of the protein sequence typically identified. These modifications were the source of the sample heterogeneity, and thus one reason for the limited success in obtaining usable crystallographic datasets from crystals of these amidohydrolases.

Of structural and functional interest was the fact that the oxidation sites were primarily located at histidine residues adjacent to Fe$^{2+}$ ions in the presumed active sites of the amidohydrolases. Excess oxidation can be avoided using an alternate expression system (e.g. baculovirus) or adding excess Mn$^{2+}$ and an iron chelator such as 2,2′-dipyridyl prior to induction during E. coli expression. In contrast, oxidation was not been observed in members of the enolase superfamily, since these proteins bind only a divalent metal ion such as Mg$^{2+}$ or Mn$^{2+}$ and not iron.

Analysis of the resulting crystallographic structures

Leverage of new crystallographic structures by modeling

To determine the impact of a structure on the structural mapping of the protein sequence space, we determine how many known protein sequences can be modeled based on the structure (i.e., the modeling leverage) (Table 2). Each enolase structure is a useful template for calculating comparative models for 2,500 to 3,200 other protein sequences in the NR database; a template is considered useful when the resulting model is based on a significant PSI-BLAST E-value (0.0001) or a favorable GA341 model score (>0.7). In contrast, the amidohydrolase superfamily structures fall into two categories: most are detectably related to 3,000–3,800 other proteins, but five structures (PDB Codes: 2I5G, 2Q01, 2Q6E, 2RAG, and 3B40) are related to a significantly smaller number of sequences (approximately 300–1,000).
A comparison of these numbers to the template-based modeling results for all NYSGXRC structures as of May 2007 (Table 5) shows that the average number of models per structure is significantly higher for the amidohydrolase and enolase superfamilies than for all structures determined by NYSGXRC (2,681 vs. 1,964). This difference reflects the relatively large sizes of the amidohydrolase and enolase superfamilies; according to the Superfamily database (http://supfam.org, [58]), across all of the superfamilies in the database, there are on average 1,770 protein sequences per superfamily.

Breaking down the modeling leverage by sequence identity reveals that the modeling leverage for the amidohydrolase and enolase superfamily structures is higher and lower than that for all NYSGXRC structures below and above the sequence identity cutoff of 30%, respectively. These differences are likely due in part to the relatively high diversity in the amidohydrolase and enolase superfamilies.

Upon initiation of the ENSPEC/NYSGXRC effort in June 2005, 45% of all known members of the amidohydrolase and enolase superfamilies were related to a known structure with a sequence identity higher than 30%. Due to the increased number of templates from the amidohydrolase and enolase superfamilies contributed by our consortia, this number increased to 73%. The total number of unique sequences modeled using the new amidohydrolase and enolase superfamily structures is 11,097, approximately 30% more than the number of known sequences from the amidohydrolase and enolase superfamilies. Among these additional sequences, we expect both members of other superfamilies with the TIM
barrel fold, as well as currently unidentified members of the amidohydrolase and enolase superfamilies, because the sequence databases have been growing by approximately 50% since 2005, and also because we concentrated on selecting only targets from the NYSGXRC genomes in the target selection process for this project.

Distribution of targets over the amidohydrolase and enolase superfamilies

For large groups of related sequences, such as the amidohydrolase superfamily network-based visualization of their relationships is helpful in generating hypotheses about how various enzymes in the superfamily evolved, and on how closely the subgroups are related to each other. We have plotted cytoscape networks for the amidohydrolase and enolase superfamilies, based on clustering by sequence similarity, and marked previously known structures, and the final targets and the structures from this project (Fig. 3). For clarity, we circled a few distinct subgroups. Another network representation with all sub-group assignments can be found in the supplemental materials.

Many subgroups in the large amidohydrolase superfamily, such as the urease-like subgroup and the uronate isomerase-like subgroup, are distinctly separated from the other superfamily members. This separation can most simply be interpreted as the result of the extreme divergence of these subgroups; thus, they are “outliers” in the overall context of the superfamily (see below for further discussion of this subgroup).

Four of the five divergent amidohydrolase structures with a considerably smaller number of homologs are separated from the main amidohydrolase network, even at the relatively non-stringent E-value cut-off of $10^{-10}$ required to visualize connections between nodes. Two of them (2Q01, 2Q6E) belong to the uronate isomerase-like subgroup. Another two of these structures (2RAG, 3B40) are clustered together with a number of unclassified sequences as well as several membrane dipeptidase-like amidohydrolase superfamily members, possibly indicating that these targets are additional members of the membrane dipeptidase subgroup. This subgroup membership is also supported by their annotation as putative dipeptidases in UniProt.

For the enolase superfamily, we chose to generate a cytoscape network that represents only four subgroups, containing the majority of the targets. The targets were mostly chosen from the mandelate racemase-like subgroup, because it is the largest subgroup with little previous structural coverage, and from the more divergent muconate cycloisomerase subgroup. The cytoscape networks illustrate that the targets and the resulting structures are indeed concentrated in regions of superfamily sequence space that lacked structural characterization prior to the start of the project, as desired for our target selection.

Examples of biological impact resulting from new structures obtained in this study

**Amidohydrolase superfamily example: atypical uronate isomerase Bh0493**

The enzymes in the uronate isomerase family are members of the amidohydrolase superfamily, although they are extremely diverged from other clusters of the amidohydrolase superfamily network (Fig. 3a). Target 9247a (gi 10173106, Bh0493) from Bacillus halodurans was identified by our structure-based expansion as a putative uronate isomerase [29]. In most organisms, both glucuronic acid and galacturonic acid are first isomerized by a single uronate isomerase, followed by further modification by several sugar specific dehydrogenases and dehydratases. In B. halodurans, as in several other organisms, two uronate isomerase genes are found, in

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**Table 5** Comparison of template-based modeling statistics for the 61 ENSPEC/NYSGXRC structures and all 327 NYSGXRC structures (May 2007)

|                                | Amidohydrolase and enolase superfamily members | All |
|--------------------------------|-----------------------------------------------|-----|
| Average number of sequences with acceptable models | 2,681                                         | 1,964 |
| Minimum/maximum number of sequences with acceptable models | 189/3693                                      | 30/6320 |
| Average number of sequences with >50% sequence identity, at least 50% coverage | 15                                            | 20 |
| Average number of sequences with 30–50% sequence identity, at least 50% coverage | 59                                            | 113 |
| Average number of sequences with <30% sequence identity, at least 50% coverage | 2,572                                         | 1,400 |

An acceptable model is defined to be based on a significant PSI-BLAST E-value (0.0001) or a favorable GA341 model score (>0.7)
operons containing dehydrogenase as well as dehydratase enzymes, consistent with this assignment of activity. We characterized both uronate isomerase genes, a “typical” uronate isomerase, Bh0705, and Bh0493, an “outlier” relative to other characterized members of this family (Fig. 4a). Although the results showed that each enzyme can isomerize both substrates, galacturionate and glucuronic acid, the Bh0705 uronate isomerase preferentially isomerizes glucuronic acid (approximately 100 times faster than galacturonic acid). In contrast, Bh0493 isomerizes glucuronic acid and galacturonic acid almost equally efficiently. These observations indicate that in *B. halodurans*, the “typical” uronate isomerase (Bh0705) has specialized its catalytic activity to preferentially isomerize glucuronic acid, perhaps because the isomerization of galacturonic acid is sufficiently achieved by Bh0493.
To gain further insight into the structural differences between Bh0493 and the “typical” uronate isomerasers (and between uronate isomerasers and other members of the amidoxydrolase superfAMILY), and in the absence of a structure of Bh0705, we compared the structure of Bh0493 (PDB codes 2Q08 and 2Q6E) to another “typical” uronate isomerase from Thermotoga maritima (PDB code 1J5S). As shown in Fig. 4b, the functionally important residues Arg170, Arg357 and His49, are conserved and cluster together within the enzyme active site both in the T. maritima enzyme and Bh0493. However, an additional metal-coordinating histidine that is usually found at the end of β-strand five in “typical” uronate isomerasers (H290 in the 1J5S) is missing in Bh0493, which has a Met (M258) in that position. The Zn²⁺ ion is coordinated by two histidine residues (His28 and His26) plus Asp355. Guided by these structures, further biochemical and computational studies to examine the differences between these two types of uronate isomerasers, and how they may be related to their different specificities, are currently in progress.

Enolase superfamily example: mandelate racemase subgroup

The SFLD currently describes 17 different families in the enolase superfamily, each performing a different overall reaction associated with different substrates and products. For the approximately 50% of the superfamily sequences whose functions are yet unknown, we estimate that roughly 15–20 novel functions (i.e. new families) will be identified. Across the superfamily, the sequences whose functions are not yet identified can be clustered into three primary subgroups and several smaller ones based on sequence and structural differences, including differences in the constellations of active site residues involved in binding specificity and catalysis [10]. In the mandelate racemase subgroup, most of the enzymes with characterized reactions are dehydratases acting on acid sugars, with the “outlier” enzyme being mandelate racemase itself. All structurally characterized members of the subgroup can be distinguished by a His-Asp dyad at the ends of β-strands six and seven that is associated with proton abstraction of substrates in the R-configuration [59]. Mandelate racemase and several acid sugar dehydratases that were previously structurally and functionally characterized also have a conserved Lys-X-Lys motif on β-strand two, with the second Lys in this motif involved in proton abstraction of substrates in the S-configuration [42]. Within this subgroup, we also observe divergence in this motif among several members of both known [32] and unknown function.

Three members of the mandelate racemase subgroup whose structures were determined by NYSGXRC, 2GL5 and 2O56 from Salmonella typhimurium and 2O4X from Zymomonas mobilis, were found to have a Lys-Val-Asp sequence motif at this position, possibly indicating a different catalytic mechanism or yet other novel function(s). The three structures align within 50% sequence identity to each other. The next closest structures (30% sequence identity) are also members of the mandelate racemase subgroup: 2POZ from Mesorhizobium loti and 2POD from Burkholderia pseudomallei have Lys-Phe-Tyr and Lys-Ile-Trp motifs at this position, respectively, providing further evidence for divergent catalytic function(s). Their structures reveal details of differences relative to that of well-characterized subgroup members containing a “canonical” Lys-X-Lys motif, providing information expected to be useful in identifying their functions. Figure 5 shows superpositions of mandelate racemase with 2GL5 and 2POD, illustrating the differences in this motif. Guided by these new structures, these enzymes are now being further analyzed computationally and experimentally.

Conclusion

The Enzyme Specificity Consortium and the New York SGX Research Center for Structural Genomics made significant progress towards characterizing the structures and functions in the amidoxydrolase and enolase superfamilies. New members of the amidoxydrolase superfamily have been identified through a combination of sequence- and structure-based expansions of the pool of known superfamily members. The structure-based expansion was particularly successful in identifying previously unrecognized superfamily members. The 63 crystallographic structures from the structural genomics pipeline increased the fraction of the sequences in these two superfamilies that can be modeled based on at least 30% sequence identity from 45% to 73%.

As an annotation tool for the targets in the two superfamilies, template-based modeling of all sequences that had detectable homology to a known structure in the amidoxydrolase or enolase superfamily allowed us to suggest previously un-annotated amidoxydrolase sequences, several of which were subsequently verified by experiment, as shown for Bh0493 in this paper. This demonstrates the power of combining sequence- and structure-based approaches for the structural genomics of two large and diverse enzyme superfamilies.
Fig. 5 Mandelate racemase bound to a substrate analog, atrolactate, (1MDR: red), is shown superimposed with two structures of unknown function. In both superpositions, active site metal ligands D195, E221, E247, the active site His-Asp dyad (H297, D270), and a Lys-X-Lys motif (K164, K166) conserved in 1MDR and other members of the mandelate racemase subgroup are labeled (1MDR numbering). a Superposition of 2GL5 (blue) with 1MDR shows conservation of all of these active site residues, except for the second Lys in the Lys-X-Lys motif of 1MDR, which is replaced in 2GL5 by Asp170. This residue faces away from the active site in 2GL5. b Superposition of 2POD (green) with 1MDR also shows conservation of all of listed residues, except for the second Lys in the Lys-X-Lys motif that is replaced in 2POD by W176.

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