Molecular function prediction for a family exhibiting evolutionary tendencies toward substrate specificity swapping: Recurrence of tyrosine aminotransferase activity in the Iα subfamily

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ABSTRACT

The subfamily Iα aminotransferases are typically categorized as having narrow specificity toward carboxylic amino acids (AATases), or broad specificity that includes aromatic amino acid substrates (TATases). Because of their general role in central metabolism and, more specifically, their association with liver-related diseases in humans, this subfamily is biologically interesting. The substrate specificities for only a few members of this subfamily have been reported, and the reliable prediction of substrate specificity from protein sequence has remained elusive. In this study, a diverse set of aminotransferases was chosen for characterization based on a scoring system that measures the sequence divergence of the active site. The enzymes that were experimentally characterized include both narrow-specificity AATases and broad-specificity TATases, as well as AATases with broader-specificity and TATases with narrower-specificity than the previously known family members. Molecular function and phylogenetic analyses underscored the complexity of this family's evolution as the TATase function does not follow a single evolutionary thread, but rather appears independently multiple times during the evolution of the subfamily. The additional functional characterizations described in this article, alongside a detailed sequence and phylogenetic analysis, provide some novel clues to understanding the evolutionary mechanisms at work in this family.

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INTRODUCTION

Subfamily Iα aminotransferases are pyridoxal 5'-phosphate (PLP)-dependent enzymes that convert an amino acid into its α-keto acid, with the concomitant synthesis of a second amino acid from its α-keto acid. The primary substrates used by this family of enzymes are aspartate, glutamate, tyrosine, and phenylalanine, and their corresponding keto acids: oxaloacetate (OAA), α-ketoglutarate (αKG), hydroxyphenylpyruvate (HPP), and phenylpyruvate (PP). The extent to which a substrate is preferred varies from enzyme to enzyme. The enzymes have been classified on the basis of this preference into two groups (Scheme 1). Aspartate aminotransferases (AATases) prefer aspartate to the aromatic substrates, while tyrosine aminotransferases (TATases; also known as aromatic aminotransferases) catalyze the transamination of the dicarboxylic and aromatic amino acids with approximately equal rate constants. Aspartate aminotransferase activity is essential due to its roles in central metabolism. OAA is an intermediate in the citric acid cycle, and Asp is an intermediate for the biosynthesis of other amino acids, nucleotides, and other metabolites. Thus interconversion of Asp and OAA connects these basic processes. In eukaryotes, AATases play a second important role in the malate aspartate shuttle; therefore both mitochondrial and cytosolic isozymes are expressed. While AATases are constitutively expressed in microorganisms such as Escherichia coli, TATases are metabolically regulated. In E. coli, TATase (eTAT) is used in the biosynthesis of Tyr and Phe as indicated by gene repression by Tyr. Conversely, the TaTase gene in Pseudomonas aeruginosa is induced by aromatic amino acids and the enzyme product (PhhC) is used in catabolism of Tyr and Phe.

AATases and TATases perform essential functions, but the AATase and TATase activities can be provided by enzymes within or outside of the Iα subfamily of aminotransferases (such as the mammalian Iy TATases). Like all members of the Family I and II aminotransferases (Pfam family PF00155), these other aminotransferases share some characteristics with the Iα subfamily aminotransferases. For example, the catalytic base is a lysine residue, which can be aligned across all aminotransferase superfamily sequences, and 11 additional residues are conserved in Family I. Yet sequence similarity studies have shown the distinct subfamilies to be distinct monophyletic clades in the phylogeny and kinetic studies have demonstrated some important differences. Many organisms possess multiple AATases and TATases in one or more subfamilies, where the redundacy provides more precise functional, temporal, or spatial control over the enzyme activities. Such complexity means that it is not certain, a priori, what the substrate specificity of an aminotransferase will be. Nonetheless, the biological data lead to certain inferences; for example, animals tend to have two subfamily Iα AATases—one cytosolic and one mitochondrial, both of which perform functions critical to metabolism—and no TATases from this subfamily.

The general molecular function of proteins in sequence databases (such as reaction specificity) is misannotated at a rate of at least 5%, while it has been estimated that about one-third of all specific annotations (such as substrate specificity) are incorrect. Annotation of the subfamily Iα aminotransferases is no exception, making accurate prediction of substrate specificities of newly sequenced genes within this family challenging. The sequences and structures of all enzymes in this subfamily are similar (>30% sequence identity; <1.8 Å r.m.s.d. of Cα atoms). Figure 1 shows the nearly superimposable active sites of 2 of the 10 aminotransferases whose crystal structures have been solved. With such high sequence and structural similarity, one may hypothesize that the proteins share a similar molecular function and possibly even substrate specificity.

The substrate preference is defined by the ratio of the specificity constants, $k_{cat}/K_m$ for each class of substrate. An aminotransferase is an AATase if its ratio for the aspartate reaction to the aromatic reaction is >1. Conversely, a ratio <1 is indicative of a TATase. For example, eAAT has a specificity ratio of 800 for aspartate to phenylalanine, while eTAT has a specificity ratio of 0.04. Yet, the sequences of these two enzymes are 42% identical. Furthermore, the PhhC sequence is more similar (46% identity) to that of eAAT than it is to the eTAT sequence (44% identity). Thus, sequence identity is

![Scheme 1](Image)

The traditional view of substrate specificity of family Iα aminotransferases. Aspartate aminotransferases (AATases) preferentially catalyze the reversible reaction on the left, while tyrosine aminotransferases (TATases) catalyze both the left and right reversible reactions with comparable rate constants. The α-ketoacids corresponding to the amino acids are oxaloacetate (OAA), α-ketoglutarate (αKG), phenylpyruvate (PP) and hydroxyphenylpyruvate (HPP).
a poor indicator of the substrate specificity within subfamily Iα aminotransferases.

The HEX design, reported by Onuffer and Kirsch, mutated the six known conserved AATase residues (as of 1993) to those found in the eTAT sequence. The substitutions sufficed to convert eAAT to an enzyme with substantial TATase activity. The HEX mutations are important in the context of eAAT as the six point mutations do not have identical effects in the presence of other scaffolds. Thus, the context of mutations is a key variable in protein redesign. Additionally, there are many solutions to the problem of converting an AATase into a TATase as illustrated by the successful conversion by directed evolution. These solutions in aggregate challenge our standard models capturing how molecular function evolves and how protein function is controlled by sequence, in that protein function does not appear to evolve in parallel with protein sequence in this subfamily. We would like to generalize these solutions to begin to understand the mechanisms of evolution and function determination. Understanding these mechanisms can ultimately be used to provide more reliable substrate specificity annotations and aid in enzyme design.

The availability of more Iα aminotransferase sequences has revealed more about the subfamily diversity. Some of the enzymes share less than 40% of their amino acid sequence with any other subfamily member with experimentally characterized substrate specificity. The full extent of diversity can be better appreciated if the substrate specificities are known at a higher resolution throughout the family. To this end, a set of diverse aminotransferases was chosen for substrate specificity characterization. We report the kinetic constants for 11 distantly related aminotransferases, and we observed that there are many instances of a single substrate specificity arising independently in the evolutionary history of this protein family. We applied a statistical model for phylogenetic-based molecular function prediction in order to elucidate the evolutionary journey of the different proteins in the aminotransferase family.

**MATERIALS AND METHODS**

Reagents were from Sigma-Aldrich (St. Louis, MO) or Fisher (Fairlawn, NJ), unless otherwise indicated.

Malate dehydrogenase (MDH) and hydroxyisocaproate dehydrogenase (HO-HxoDH) were prepared as described previously, except that HO-HxoDH was expressed in Rosetta(DE3)pLysS cells (EMD, San Diego, CA) from the plasmid pHisH described below. The cloning, expression, and purification of aminotransferases are described elsewhere.

Subcloning of HO-HxoDH

All enzymes used for cloning were from New England Biolabs (Ipswich, MA) except that alkaline phosphatase was obtained from USB (Cleveland, OH). Purification of DNA fragments was carried out using GFX kits from GE Healthcare (Piscataway, NJ).

pHisH was made by subcloning the HO-HxoDH gene from the pTrc-99a construct, pHicDH-His1, described in Aitken et al., into pET19b (EMD) to increase expression levels. pHicDH-His1 does not have the unique restriction sites necessary for direct cloning
into pET19b, therefore an extra subcloning step was undertaken to introduce a new restriction site. pHiCDH-His1 was sequentially digested with NcoI and XbaI restriction enzymes, and the ~1000 base pair fragment from the pHiCDH-His1 digestion was gel purified. This purified fragment was ligated to XbaI-digested pET19b with T4 DNA ligase. This last step inserted an adapter sequence between the gene and vector—adding a BamHI restriction site downstream of the HO-HxoDH gene—and produced a linear, not circularized, product. The product was digested with BamHI and a ~1000 base pair fragment, corresponding to the HO-HxoDH gene with a sticky NcoI 5’ end as well as a sticky BamHI 3’ end, was gel purified. More pET19b was digested with NcoI and BamHI, treated with shrimp alkaline phosphatase and a ~5000 base pair fragment was gel purified. Finally, these two fragments were ligated to make pHicHis.

The plasmid was transformed into E. coli strain DH10B (Invitrogen, Carlsbad, CA) by electroporation with a Bio-Rad (Hercules, CA) GenePulser. DNA plasmid purification was done with a Wizard Midiprep kit from Promega (Madison, WI). The product was confirmed by DNA sequencing performed by Elim Biopharmaceuticals (Hayward, CA).

Kinetic assays and data fitting

AATase activity was measured by MDH-coupled assays containing 200 mM TAPS, pH 8.0, 100 mM KCl, 150 μM NADH, and 10 μM PLP. Aspartate and αKG concentrations were varied. TATase activity was measured by HO-HxoDH-coupled assay containing 100 mM TAPS pH 8.0, 100 mM KCl, 150 μM NADH, and 10 μM PLP, while concentrations of Phe and αKG were varied. Activity with isoleucine, leucine, tyrosine and valine as substrates were measured with the same coupled assay. The rates of product formation were measured by loss of NADH absorbance at 340 nm. All measurements were made on an Agilent 8453 UV-Vis spectrophotometer or SpectraMax 190 UV-Vis plate-reader (Molecular Devices).

Kinetic data were fit with either the SAS (SAS Institute, Cary, NC) or Origin applications (OriginLab, Northampton, MA) to Eq. (1) describing a ping-pong bi-bi reaction:34

\[
\frac{v}{K_m} = \frac{k_{cat}[E][AA][\alpha KG]}{K_m^{AA}[\alpha KG] + K_m^{KC}[AA] + [AA][\alpha KG]}
\]

(1)

where [E] and [AA] are the concentrations of enzyme and amino acid substrate, respectively. Equation (1) reduces to:

\[
v = \frac{k_{cat}[E][AA]}{K_m}
\]

(2)

where \(K_m^{AA} \gg [AA]\). Equation (2) was used to fit the data when saturating concentrations of amino acids could not be attained.

Manual selection of aminotransferases

UniProt35 was queried for all sequences containing the keyword “aminotransferase” (1726 entries, as of April, 2003). The sequence alignment software, SATCHMO, was designed to align sequences with low pairwise similarity as well as those with higher overall sequence similarity but local variance in sequence.36 As pairwise similarity increases and local variance decreases, SATCHMO’s alignment improves. However, it has a built-in limitation on the memory requirements for alignment, which, in practice, meant that only about 50 divergent aminotransferase sequences could be aligned by SATCHMO at a time. Therefore, the 1726 aminotransferase sequences were arbitrarily divided into 32 batches, each containing approximately 50 sequences.

In order to identify aminotransferases that were likely to be in the Iα subfamily, all sequence batches were iteratively aligned to each other and to two subfamily Iα reference sequences, cPigAAT and eAAT, with SATCHMO (note that cPigAAT and eAAT aligned well with each other as determined by visual inspection). Sequences were eliminated if they did not contain a lysine that aligned to the active site lysine of cPigAAT (K258*) according to SATCHMO’s indication of alignable columns or if the alignment failed to converge (10 batches). This first round eliminated > 80 % of the sequences, leaving 325 sequences aligning with K258 of cPigAAT. These 325 sequences were arbitrarily divided into seven smaller batches and aligned under the same criteria, eliminating an additional 83 sequences. A third round was completed as a single batch with 242 remaining sequences and with the minaff option set to ~0.5 because the method failed to converge with the default setting due to sequence divergence; 55 sequences were eliminated in this round. Analysis of the Swiss-Prot annotations and corresponding primary literature for the remaining 189 sequences revealed that all known subfamily Iα aminotransferases were localized to a distinct clade of 92 sequences in the tree produced by SATCHMO. The final SATCHMO alignment of these 92 subfamily Iα sequences was manually refined based on a structural alignment produced by MAPs37 of PDB entries 1AJS (cPigAAT), 2CST (cChickAAT), 1ASM (eAAT), 1MAP (mChickAAT), 3TAT (eTAT), 1AY5 (PdTAT), and 1YAA (SccAT).

This alignment of 92 sequences was used as the foundation for selecting a group of divergent proteins for kinetic characterization. Briefly, the sequences were grouped according to their similarity near the active site, and then a representative enzyme from each group was selected for further study. The unliganded eAAT crystal structure (PDB code 1ASN) was used to identify residues near the active site, defined here as being <15 Å from the nearest atom of the PLP cofactor. Moderate

*Chicken cytosolic AATase numbering
variability was determined from the overall percent conservation at a given position observed in the SATCHMO alignment of 92 sequences. For the purposes of this study, a residue has moderate variability if it is the same amino acid in at least 25%, but fewer than 75%, of the aligned sequences. Seventy-six positions out of ~400 met the distance and variability (D&V) criteria, which we defined as <15 Å from cofactor and 25 to 75% identity. Each of the 92 subfamily Iα sequences in the SATCHMO alignment was compared with the set of 10 kinetically characterized reference sequences at each of these 76 positions. The latter reference set includes: (1) the proteins listed in Table I, which is a comprehensive set of class Iα aminotransferases for which there exists published kinetic data for aspartate and at least one of the aromatic substrates; (2) *Saccharomyces cerevisiae* cytosolic aspartate aminotransferase (SccAT), which has a published crystal structure; and (3) *P. aeruginosa* aspartate aminotransferase (PaAT).

For each position chosen using the D&V criteria, a sequence’s score (the D&V score) increased by one for each residue that was different from the corresponding residues in all the 10 characterized reference sequences in the given alignment. The total possible D&V score was 76, based on the total number of chosen residues in these sequences. Most of the sequences were similar or identical to those that had been previously characterized and consequently had D&V scores of 10 or less. A smaller set of thirty-two sequences with a D&V score > 10, and, therefore, greater sequence diversity near the active site, were carried forward for further analysis. A pair-wise score was calculated for each of these 32 top-scoring sequences to create a distance matrix in order to group the sequences according to their relative divergence. To compute the pair-wise score, two sequences were compared at each position that contributed to the original D&V score in that sequence, and one was added to the pair-wise score for each residue that was mismatched between the two sequences. These pair-wise scores are not necessarily symmetric since the positions contributing to the original D&V score may be different for each sequence. Pairs of sequences where both members of the pair score <9 relative to each other were placed into the same group (Fig. 2). One enzyme from each of these 10 groups was chosen for characterization based on gene availability. Thus, the active site of each enzyme that was selected was different from the 10 original reference enzymes, and also different from each of the other nine newly selected enzymes.

Using the D&V scores, we selected 10 distantly related aminotransferases that were previously uncharacterized to subject to kinetic analysis. As reported previously, attempts to obtain pure *Arabidopsis thaliana* cytosolic aminotransferase (AtcAT) were unsuccessful and the enzyme could not be characterized, but is included in the phylogenetic analyses here. The yeast cytosolic aminotransferase was also characterized because, while its crystal structure was solved, there are no reports in the literature of its kinetic activity with aromatic substrates. Kinetic data are also presented for the first time for PaAT, bringing the total number of Iα aminotransferases characterized here to 11.

### Phylogenetic analysis: The SIFTER method

The Statistical Inference of Function Through Evolutionary Relationships (SIFTER) method was applied to the aminotransferase Iα subfamily. We reconstructed a phylogenetic tree of the 92 Iα sequences identified using the iterative SATCHMO alignment method above. The 92 Iα sequences were aligned to 41 Iγ sequences with MUSCLE and manually reconciled to the structural alignment described above (MAPS alignment of seven Iα structures). A phylogeny was built from this alignment with RAxML, a fast, maximum likelihood method for reconstructing phylogenies, with 100 iterations of bootstrapping. The Iγ sequences were used as outgroup references to ensure proper rooting of
the tree. A final consensus tree was created by the Consense program from the Phylip package with rooted trees.\(^4^4\) The subfamily phylogeny is shown in Figure 3.

We ran SIFTER 2.0\(^4^5\) on the phylogeny of 92 proteins belonging to the \(I\alpha\) subfamily in two ways: either including as input to SIFTER the existing set of eight functional characterizations (Table I), or including the 19 existing and new functional characterizations. In both cases, SIFTER produced a set of molecular function predictions for the proteins that did not have functional annotations as input. These results were used to perform a phylogenetic analysis of the family, and to compare phylogenetic analyses before and after the addition of the new functional annotations. We also performed leave-one-out cross-validation for both the existing set of functional characterizations and the existing and new functional characterizations to determine how the additional data improved predictions for uncharacterized proteins in this family. Leave-one-out cross-validation removes a single protein’s experimental annotation and then predicts the annotation for that protein using only the remaining annotations.

### RESULTS

#### Aminotransferase identification and alignment

The motivation for this research grew from three related goals: (1) to facilitate the prediction of function of uncharacterized aminotransferases from the \(I\alpha\) subfamily, (2) to identify the substrate specificity determinants, or the residues in the active site that play major roles in specificity and (3) to identify where and how substrate specificity is determined in the evolutionary history of this family using a phylogenetic analysis. The initial objective was then to gather substrate specificity data for a representative group of subfamily members to enable an informed phylogenetic analysis.

The construction of the set of broadly representative \(I\alpha\) aminotransferases was guided by the objective of obtaining a large cross-section of possible active sites that have AATase or TATase activity with the backbone of the \(I\alpha\) subfamily. A fingerprint of the conserved residues for this subfamily, which was defined by Jensen and Gu,\(^5\) was based on the limited set of \(I\alpha\) subfamily member protein sequences available before 1996. This
Dendrogram of subfamily Iα aminotransferases. The rooted tree of Iα aminotransferases was created with RAxML and the Consense application in the Phylip package using Iγ aminotransferases for the outgroup (outgroup not shown in figure for brevity). Branch length values are indicated on branches, but are omitted from select branches for clarity. The species and UniProt identifiers are indicated on each leaf (UniProt accession numbers corresponding to the Swiss-Prot sequences are in Supporting Information Table S1). Confirmed AATase and TATase annotations are highlighted in cyan and magenta, respectively, and AtcAT, for which kinetic data was not successfully obtained, is outlined in black. The 11 enzymes that were kinetically characterized in this work are indicated by an asterisk (*) to the right of the leaves.
The UniProt database contains Swiss-Prot, a manually curated database, and TrEMBL, which is a computer-generated compilation of other databases, including GenBank. Since the objective was to cover the breadth of protein sequence and function, not to gather the largest possible data set of sequences, the UniProt database was probed for probable aminotransferase sequences. The breadth of sequence and function coverage for full-length enzymes in the UniProt database is comparable to GenBank; Swiss-Prot annotations are, overall, more accurate. A full-text keyword search of UniProt for entries for “aminotransferase” yielded 1736 sequences that are potentially members of all aminotransferase families (as of publication, close to 110,000 entries now contain this keyword, consistent with general growth trends of UniProt). This sequence set was manually pruned by comparison to the sequences of two known Iα aminotransferases, cPigAAT and eAAT, in order to identify the likely Iα aminotransferases. An alignment of a similarly distant set of 20 Iα aminotransferases (Fig. 4) illustrates that the subfamily sequences align well and, despite the fairly large number of amino acid substitutions, some highly conserved regions are maintained across the subfamily.

The most reliable family I aminotransferase identifier is the sequence location of the active site lysine. From the pruned set, 189 sequences aligned at this locus with the cPigAAT K258 in multiple rounds of batch alignment (see Materials and Methods for details). Analysis of the Swiss-Prot sequences and their positions in the dendrogram calculated by SATCHMO revealed separate clades for the Iα subfamily, the histidinol-phosphate aminotransferase subfamily (Iβ), the Iγ AATases and TATases, and alanine aminotransferases (Iβ6). This result is consistent with prior phylogenetic characterizations of these subfamilies. The final subfamily Iα clade contains 92 sequences, not all of which are unique (Fig. 3). For example, there are three nearly identical sequences from E. coli: Swiss-Prot ID AAT_ECOLI, and UniProt AC Q8XDF3 and Q8FJ99, two of which are probably either population variants or sequencing errors.

While the first shell of residues around the active site in aminotransferases makes important contacts with the substrate and cofactor, PLP, second and third shell residues have also been shown to play roles in substrate specificity. All residues are within 32.2 Å of a PLP atom in the unliganded eAAT structure (PDB code 1ASN), and those that are three shells away from the PLP are within 16.3 Å, while those that are four shells away are ≤22 Å from a PLP atom (i.e., approximately 5 Å per shell).

To quantify the conservation of amino acids around the active site, we collected the set of sixteen amino acids that are ≤3.40 Å from the PLP (cofactor) or maleate (ligand) in the eAAT structure (PDB code 1ASM). The 16 residues in this first shell are: Ile17, Gly38, Tyr70, Gly108, Thr109, Trp140, Asn194, Asp222, Ala224, Tyr225, Ser255, Ser257, Lys258, Arg266, Arg292, and Arg386 (shown in Figs. 1 and 4). The quality score, or q-score, in the ClustalX alignment software for each of these columns denotes the level of similarity within that column of the alignment, with a value of 100 meaning that the amino acid is completely conserved across all of the sequences and a value of 0 indicating that the amino acid is not conserved at all. The sum of the q-scores for these 16 active site residues was 1446 (1600 maximum) using the alignment shown in Figure 4. To check whether the amino acids involved in the binding site are conserved relative to the remaining amino acids in this protein, we performed a permutation test by sampling randomly without replacement from all the columns in the alignment for which there was not a gap in the eAAT sequence. This test yields a significant P value (<10−5) indicating that the residues near the active site are significantly more conserved than residues chosen at random in this alignment. In particular, while the sum of the q-scores of these 16 columns in the alignment is 1446, the largest q-score sum of 16 columns randomly sampled without replacement 100,000 times was 1107. This level of conservation relative to overall sequence conservation in this family of proteins implies that these 16 amino acids are important for aminotransferase function. The results from the permutation test and the observations specific to the aminotransferase subfamily suggest that residues that are moderately conserved and near the active site are most likely to play key roles in substrate specificity.

A goal of using the D&V scoring method was to select 10 new aminotransferases to characterize, effectively doubling the kinetic data for this subfamily. As described in Materials and Methods, all identified Iα aminotransferases (92 sequences) were compared with a set of 10 kinetically characterized reference aminotransferases at each of the 76 residues selected based on the distance and variability (D&V) criteria (see Materials and Methods for details). If overall sequence identity had been used as the selection criterion instead of a D&V method, a cut-off of 65% identity would have selected 12 sequences, in which each sequence is <65% identical to all of the kinetically characterized reference sequences and also <65% identical to each of the other 11 new sequences. In this scenario, while Groups 1, 3, 5, and 7 to 10 in Figure 2 would each be represented with one sequence and Group 2 with two sequences, Groups 4 and 6 would be eliminated and therefore no plant cytosolic or mitochondrial enzymes would have been chosen for characterization. The remaining 3 of these 12 sequences...
received low scores by the D&V method (a low score means high similarity to the reference set of sequences). Schizosaccharomyces pombe O94320 is the least similar of the three to the reference aminotransferases with a D&V score of 9 out of 76, while the other two are quite similar to the previously characterized set: their scores are both 4.

**Kinetic characterization**

The kinetic constants characterizing the transamination of aspartate and phenylalanine for 11 aminotransferases, as compared with a representative AATase and TATase, are presented in Table II. *Caenorhabditis elegans* cytosolic aminotransferases. The kinetic parameters for the AATases, coded by the top four sequences, were determined earlier, and the bottom four are characterized TATases. The substrate specificities and kinetics of the remaining 12 enzymes were determined in this study. The sequences are ordered alphabetically within each group. The boxed sequence, AtcAT, has unknown substrate specificity (see Materials and Methods). The sequences above the box are now assigned as AATases, and those below are TATases. The alignment numbering is based on *cChickAAT*. The sequences were aligned by MUSCLE, with manual refinement based on a structural alignment produced by MAPS. The 23 positions highlighted in black are completely conserved in subfamily Ia aminotransferases. This is a reduction from the 51 specified in Jensen and Gu. The 16 first-shell residues (≤3.4 Å from the cofactor or inhibitor) are marked with an asterisk.

### Figure 4

Sequence alignment of subfamily Ia aminotransferases.
Figure 4

Continued
AATase (CecAT) displays the strongest preference yet demonstrated for aspartate, with a specificity constant ($k_{cat}/K_m$) ratio of aspartate to phenylalanine of 80,000. Most enzymes with a preference for aspartate (A. thaliana mitochondrial AATase (AtmAT), CecAT, Chlamydia trachomatis AATase (CtAT), Plasmodium falciparum cytosolic AATase (PfcAT), PaAT, SccAT, and S. cerevisiae mitochondrial AATase (ScmAT)) have $K_m^{Asp}$ values of about 1 to 3 mM, and $K_m^{Phe}$ values $> 30$ mM. The exception is Trypanosoma brucei mitochondrial AATase (TbmAT), which is a poor aminotransferase with high $K_m$ values for all tested substrates (Asp, Phe, and Tyr). The kinetic constants for the transamination of tyrosine are comparable to those for phenylalanine for each of the four tested enzymes: Giardia intestinalis cytosolic TATase (GicAT), PfcAT, T. brucei cytosolic TATase (TbcAT) and TbmAT (data not shown).

All the enzymes, including the three with preferences for phenylalanine over aspartate (GicAT, TbcAT, and V. cholerae TATase (VcAT)), exhibit low specificity ratios. VcAT has the lowest specificity ratio (0.010), which accounts for most of the effect on the specificity.

### Sequence similarities and differences

About half of the 51 positions that Jensen and Gu identified as invariant in Ia aminotransferases remain conserved in the aminotransferases characterized to date (Fig. 4). Some of the invariant residues described by them, which are not conserved in the alignment of the set of sequences used here, can be explained by conservative substitutions found in this expanded set of sequences. For example, residue 140, which forms a key interaction with the pyridine ring of PLP, is either the expected tryptophan or is a tyrosine. A subset of these twenty-three completely conserved residues located between residues 194 and 386 was used for a fingerprint search of the nonredundant sequence database with the BLAST program Seedtop (available from NCBI). Conservative, infrequent substitutions are also found in the full alignment of the resulting 2635 aminotransferase sequences for key positions such as 140.

The average distance of the closest atom of the twenty-three conserved residues from the most proximal atom of PLP (based on the eAAT structure 1ASN) is 7.3 Å; compared with an overall average distance of 16.1 Å (nearest atom to nearest atom) for all residues. Ten of these conserved residues are in direct contact with either PLP or with the ligand, maleate (based on the complexed structure 1ASM), and 15 are within the first two shells of active site residues (Fig. 1). Six of the conserved amino acids are glycine, and an additional six are conserved either as lysine or as arginine. These numbers are greater than what is observed among other sets of orthologous sequences for key positions such as 140.

### Table II

Kinetic Constants for Newly Characterized Subfamily Ia Aminotransferases

| Protein | $k_{cat}/K_m^{Asp}$ | $k_{cat}/K_m^{Phe}$ | $K_m^{Asp}$ | $K_m^{Phe}$ | $k_{cat}/K_m^{Asp}$ | $k_{cat}/K_m^{Phe}$ |
|---------|---------------------|---------------------|-------------|-------------|---------------------|---------------------|
| aAAT    | 1.79                | 0.48                | 156         | NS          | 90,800              | 1.7                 |
| eTAT25  | 3.8                 | 0.90                | 140         | 37,000      | 0.26                | 250                 |
| AtmAT   | 2.5                 | 2.2                 | 89          | 36,000      | 1.7                 | 260                 |
| GicAT   | 1.3                 | 0.25                | 45          | 34,000      | NS                  | 8.8                 |
| CtAT    | 2.3                 | 0.58                | 86          | 37,000      | NS                  | 4.5                 |
| PfcAT   | 0.8                 | 0.04                | 36          | 35,000      | NS                  | (0.05)              |
| PaAT    | 2.01                | 0.02                | 99          | 47,000      | NS                  | (5)                 |
| SccAT   | 2.7                 | 0.2                | 168         | 63,000      | NS                  | 80                  |
| ScmAT   | 1.3                 | 0.1                | 18          | 14,000      | NS                  | 12,000              |
| TbcAT   | 9.6                 | 0.54                | 105         | 11,000      | 3.1                 | 4.0                 |
| TbmAT   | NS                  | (0.05)              | (4)         | (1,000)     | (0.4)               | (0.2)               |
| VcAT    | 34                  | 1.60                | 22.9        | 680         | 46                  | 67,000              |

*Conditions: pH 8.0 in 200 mM TAPS buffer and 100 mM KCl at 25°C, except TbmAT assays were done at an ionic strength = 0.43. Standard errors are in parentheses.

†NS, no saturation was observed with 40 mM of the specified amino acid substrate. $k_{cat}/K_m^{Asp}$ was determined with constant $[aKG] > K_m^{Asp}$ for the aspartate reaction. The $[aKG] = 10$ mM for the TbmAT assays.
arginines in the aminotransferases are known, the transferase active sites versus 8.0% and 7.1% for each lysines and arginines, respectively, conserved in amino-acids. 13.2% glycine conservation overall; 8.7% and 17% of are conserved in the active site of aminotransferases versus the uncharacterized sequence (AtcAT). Residues in bold type differ from the diagram presented in Rothman and Kirsch. published) was used to perform the sequence analysis to generate this figure.

Figure 5

Venn diagram of conserved residues in AATases and TATases. Those conserved in ≥75% of the sequences for each substrate specificity were identified for the sequence alignment presented in Figure 4, excluding the uncharacterized sequence (AtcAT). Residues in bold type differ from the diagram presented in Rothman and Kirsch. The Venn diagram of Figure 5 shows that 71 residues are conserved in at least 6 of the TATases (Set \( TAT \)) and 21 are conserved in at least 9 of the AATases (Set \( AAT \)). The magnitude of these differences is expected given the different amounts of substrate specificity data available for each of the two analyses. However, until we are closer to discovering the mechanism of substrate preference, it is difficult to speculate on relative similarity based on the sparse available data.

Protein function prediction

We ran SIFTER on the phylogenetic tree for the aminotransferase family containing 92 sequences (see Materials and Methods). In all experiments on this family, there were exactly two candidate functions from the Gene Ontology, L-aspartate:2-oxoglutarate aminotransferase activity (GO:0004069) and L-tyrosine:2-oxoglutarate aminotransferase activity (GO:0004838), corresponding to AATase and TATase activity, respectively. Using the default fixed parameters for SIFTER, we performed leave-one-out cross validation, including only the eight experimental annotations known before the experiments discussed here (those listed in Table I). SIFTER achieved 82% accuracy (9 of 11 substrate specificities were correct) in predicting the substrate preference of the newly evaluated enzymes; the substrate specificities of 2 of the 11 subsequently characterized sequences were predicted incorrectly (GicAT and TbcAT). We also performed leave-one-out cross-validation with the eight existing annotations plus the 11 additionally characterized proteins, for a total of 19 proteins, using the default SIFTER parameters, in order to determine if the additional characterizations improved prediction accuracy in this protein family. The additional data increased the accuracy slightly to 84% accuracy (16 of 19 correct substrate specificity predictions).

SIFTER and other methods for phylogenetic-based prediction of protein molecular function make the assumption that sequences that are closer in a phylogeny will tend to have more closely related substrate specificities. We see that this assumption is violated in this family: Figure 3 shows that there are at least three (or possibly more) locations in the phylogeny where the substrate specificity independently mutated to include aromatic amino acids. As a result, TATases appear to cluster in distant tree clades, and prediction accuracy is negatively impacted. Furthermore, additional data does not improve predictions substantially, as this assumption with our observations from our more diverse set. However, they observed a nearly equivalent number of conserved residues with either specificity (\( |AAT \cap TAT| \approx |TAT \cap AAT| \)), while our new set shows that the AATases are more similar to each other than the TATases are to each other (\( |AAT \cap TAT| = 39 \) as compared with \( |TAT \cap AAT| = 21 \) conserved amino acids). The phylogenetic-based analysis described below is in accord with these observations.

Using a less diverse set of aminotransferases, with a smaller percentage that had been kinetically characterized, Rothman and Kirsch earlier found that the putative AATases are more similar to the putative TATases than they are to other AATases, and vice versa, consistent
is violated in the newly characterized proteins according to this phylogeny just as in the previously characterized proteins.

A more sophisticated protein function prediction method might recognize this as convergent evolution and reduce the confidence values for predictions of specificities that arise independently in multiple places in the phylogeny. SIFTER includes functionality to estimate model parameters, including relative rate of convergent evolution; however, given the small number of observations and the number of parameters to estimate, we did not estimate model parameters in this application. Further, overlaying the relative activity numbers onto the phylogenetic tree does not improve the predictive power: the $k_{cat}/K_m$ specificity ratios for Asp:Phe do not cluster within the phylogeny (data not shown). Another possible route is to consider relevant motifs instead of the full sequence in the phylogenetic framework to improve prediction.

**DISCUSSION**

**Selection of diverse enzymes**

Enzymes were selected for characterization with the intended goal of finding those with divergent substrate specificity constants. The manual scoring algorithm presented here weighs sequence differences among the active site residues more heavily than those outside the active site in order to identify mutations that may have led to changes in substrate specificity (see description of D&V scoring in Materials and Methods.).

Table I lists the pairwise sequence identities of all the aminotransferases for which a definitive substrate specificity can be assigned. It is apparent from this table that overall sequence identity is not a reliable indicator of substrate specificity for aminotransferases. Choosing enzymes to characterize based on low overall sequence identity to the reference set would yield a collection with more variability in overall structure than in active site structure and substrate specificity. Within a family of proteins, those with the lowest overall sequence identity may simply have different folding or solubility requirements.

Table I indicates that AATase to TATase or vice versa specificity switching may have happened repeatedly in the evolution of the aminotransferase family. Two enzymes with high overall sequence identity may have different substrate specificities; thus, differences in active site residues should be weighed more heavily in the selection of new aminotransferases for characterization.

**Substrate specificity**

Before this work, only three AATases and two TATases had been fully characterized kinetically. These AATases exhibit specificity ratios $(k_{cat}/K_m)_{Asp}/(k_{cat}/K_m)_{Phe}) > 900$, while this value is $<1$ for the two TATases (Fig. 6). Thus the defining parameter for the specificity assignment was unambiguous. Five of the seven newly characterized AATases exhibit specificity constants $>900$, but that for CtAT is 500 (Fig. 6 and Table II). TbmAT, although quite inactive (Table II), has a specificity ratio of only 6, and its specificity assignment is both more nuanced and less certain (see below). Two of the three novel TATases (TbcAT and GicAT) have specificity ratios between those of the previously characterized PdTAT and eTAT, while the specificity ratio for VcAT is the most discriminating class Ia aminotransferase for Phe over Asp yet found (specificity constant $= 0.010$). Thus the empirical criterion revealed by the limited set of available data continues to be valid. The same sharp drop in the specificity ratio separating the near continuum of AATase values from TATases (Fig. 6, vertical line) persists, except for that of TbmAT.

The TATases, GicAT, TbcAT and VcAT, have much higher $K_m$ values for Asp than does either eTAT or PdTAT. The most striking example is VcAT, with a $K_m$ of 34 mM for Asp, which is much higher than the intracellular Asp concentration of about 0.6 mM in *E. coli*.28

**Table III**

Comparison of HEX Residues in Characterized Aminotransferases

| Position | HEX mutation $^b$ | PdTAT$^c$ | SmTAT | PhcH | Eukaryotic$^d$ |
|----------|-------------------|-----------|-------|------|----------------|
| 39       | V→L               | V         | V     | V    | A              |
| 41       | K→Y               | K         | K     | K    | R              |
| 47       | T→I               | T         | T     | T    | P              |
| 69       | N→L               | T         | A     | S    | E              |
| 109      | T→S               | T         | S     | T    | T              |
| 207      | N→S               | F         | M     | T    | N              |

$^a$Underlined amino acids indicate that the identity is switched relative to what would be expected from the HEX construct. Amino acids in bold font are the same as that predicted from HEX.

$^b$eAAT residue mutated to its analogue in eTAT.26

$^c$PigAAT, cChickAAT, and mChickAAT.

Table III lists the differences at these six positions. With few exceptions, the amino acids observed in the homologs are not what the HEX experiments predict. Other research suggests that important specificity determining residues are more highly conserved in AATases than in TATases.28 In comparison with overall sequence identity criteria, the D&V method focused our selection on differences in the active site residues, which we argue are more likely to define the enzyme's substrate specificity in this subfamily.
analyses were not carried out in the earlier study; thus, less accurate kinetic constants might have been obtained.

Results in Berger *et al.* 60 show that N-terminal His₆-tagged GicAT, PfcAT, TbcAT, and TbmAT transaminate several amino acids, suggesting that these enzymes exhibit very broad substrate specificity and function in methionine regeneration *in vivo*. A ια aminotransferase from *Leishmania mexicana*, which has high sequence similarity to TbcAT, was also shown to transaminate methionine, aspartate, and phenylalanine, among other substrates. 61 Malashock and Kirsch observed transamination of methionine by C-terminal His₆-tagged GicAT (unpublished data) although recent specific activity data for C-terminal Strep-tagged PfcAT shows no activity toward methionine. 21

The kinetic constants for aromatic amino acid transamination in Table II cannot be appropriately compared with the previous report as different co-substrates were used. Nonetheless, the present findings that GicAT and TbcAT are TATases are consistent with the overall conclusion in Berger *et al.* 59 that these enzymes are broadly specific, and may play a key role in methionine recycling. The *in vivo* function of these aminotransferases is yet to be elucidated; therefore we tentatively classify them as TATases, in order to be consistent with the current nomenclature conventions. However, if the primary function of these proteins is found to be in methionine recycling, rather than aspartate or tyrosine/phenylalanine metabolism, this assignment should be revisited.

The major role of mitochondrial aminotransferases is in the malate-aspartate shuttle; therefore, they should exhibit strong preferences for aspartate and glutamate over other substrates. AtmAT, ScmAT, and TbmAT were annotated as mitochondrial enzymes because they have longer N-termini, as found for other nuclear-encoded aminotransferases from that organelle (i.e., mChickAAT vs. cChickAAT). 59, 62, 63 Morin *et al.* noted that a signal sequence similar to that of ScmAT is present on the N-terminus of a mitochondrial alcohol dehydrogenase. 62, 64 Mitochondrial signal sequences are cleaved *in vivo*; therefore kinetic data collected on enzymes with intact N-termini may not reflect *in vivo* functionality. Nonetheless, the substrate specificity ratios should not be significantly affected by the presence of the signal sequence.

AtmAT and TbmAT were characterized with intact signal sequences, while ScmAT was characterized without its putative signal sequence. AtmAT and ScmAT have reasonable *k*₅ values for the aspartate reaction (89 s⁻¹ and 18 s⁻¹, respectively), while TbmAT exhibits low activity toward Asp, Phe, and Tyr ( *k*₅ values are from 22 to 132 M⁻¹ s⁻¹; tyrosine transamination data not shown). This *k*₅ value for AtmAT is about half of 205 s⁻¹, the value that was previously published for N-terminally truncated AtmAT. 65 Although the kinetics of truncated AtmAT were determined from linear regression at a
single concentration of co-substrate, that alone cannot explain the large difference in $K_m^{\text{Asp}}$ presented here (Table II; 2.2 mM vs. 0.26 mM). The $K_m^{\text{Asp}}$ values are similar for the full-length and truncated forms (2.5 mM vs. 3.0 mM) and, consequently, the specificity constants for Asp for the two forms of AtmAT are within a factor of 2.

The specificity ratio of Asp to Phe for AtmAT is 4100 and for ScmAT is 5000, consistent with AATase annotations (i.e., $\gg 1$). In contrast, the specificity ratio of TbmAT is only 6; it is an AATase, but does not discriminate well between substrates. While AATases do have a well-known function in mitochondria, no function is known for a TATase in that organelle; thus, the lack of specificity of TbmAT for aspartate is unexpected. The mitochondrial signal sequence or lengthy purification process may be responsible for the low activity of TbmAT, but it is also possible that it is neither an AATase nor a TATase and that the true substrate has not been identified.

**Phylogenetic analysis**

The set of 19 characterized enzymes, including the 11 presented here, are scattered throughout the subfamily Iα phylogeny (Fig. 3), even though the overall sequence identities and phylogeny were not considered in their selection. The previously characterized aminotransferases are localized to certain sections of the phylogeny, and the new characterizations fill in some, but not all, gaps.

The application of phylogenetic methods to protein function determination is predicated on the assumption that molecular function (including substrate specificity) evolves in parallel with sequence. This family shows more independent changes in substrate specificity than are suggested by the evolutionary distances. Thus, in order to localize those independent substrate preference mutations precisely within the phylogeny, it appears that more experimental data are needed, or alternative methods for protein function prediction are required. The current set of annotations shows that there are multiple instances of mutation in the tree, but there are insufficient characterizations to localize these mutations to a single branch. Multiple instances of independent, parallel evolution do not preclude a phylogenetic-based analysis.

Despite these frequent function changes, SIFTER predicted 16 of the 19 specificities correctly. With only the previously-known annotations, SIFTER predicted 9 of the 11 specificities correctly, so the analysis maintains a low cross-validation error rate with the additional protein characterizations. This number is indicative of how complete the current information is to predict substrate specificity of all of the remaining members of the tree and to enable localization of the substrate specificity mutation events. Thus, we can quantify the progress made in predicting substrate preference for uncharacterized proteins and also in localizing mutation events in the phylogeny with these characterizations.

A phylogenetic analysis suggests a series of hypotheses about this subfamily of proteins. SIFTER predicts that the root node of the phylogeny is an AATase, both before and after inclusion in the phylogeny of the set of proteins experimentally characterized here. This implies that the ancestral protein in this family may have had a preference for aspartate and that a preference for tyrosine is a more recent development, in agreement with prior analysis (although other research suggests that the ancestral enzyme had broad specificity that was subsequently narrowed). The hypothesis that the ancestral Iα enzyme was an AATase is further supported by an analysis based on parsimony: if we assume that this phylogeny has the minimal number of changes in substrate preference (i.e., three), this is only possible given the current annotations and assuming the phylogeny is accurate when the protein at the root node is an AATase. If we consider TATase activity to be at the root of the tree and AATase activity independently became the dominant function, it would have required at least five separate instances of substrate preference switching to explain the current configuration. It is quite possible that additional enzyme characterizations will increase this number, because of the large number of remaining uncharacterized subfamily members.

Because of the diversity of organisms in this subfamily phylogeny, another hypothesis we can make is that a subfamily Iα AATase may have been present in the common ancestor of bacteria and eukaryotes (representatives from archaea are notably absent). It is possible that bacteria and eukaryotes both require at least one AATase as no major lineage shows evidence of a deletion of this enzyme. The AATases of higher organisms cluster well near the top of the phylogeny in Figure 3, with mitochondrial forms in the lower clades in this region, and we can see that the protozoan aminotransferases either cluster in the middle or are segregated from the bulk of the tree (e.g., PfAT at the bottom of Fig. 3). A fingerprint search of the gene databases (based on the new sequence alignment in Fig. 4) supports the hypothesis that animals, plants, and fungi express at least two Iα AATases, corresponding to cytosolic and mitochondrial localization, with some plants and fungi expressing even more Iα aminotransferases as exemplified by *A. thaliana* and consistent with plant robustness and redundancy findings (data not shown). In contrast, protozoa may have only one Iα aminotransferase, either a cytosolic or mitochondrial AATase, and any other Iα aminotransferases may have broader substrate specificity. Finally, bacteria tend to have two Iα aminotransferases, representing each of the two specificities, although there are exceptions (e.g., the *C. trachomatis* genome encodes only one: CtAT (data not shown)). These same trends are observed in the more limited phylogeny presented in Figure 3.
CONCLUSION

The subfamily Iα aminotransferases characterized here prove to be diverse evolutionarily and also in terms of substrate preferences. Phylogenetic analysis illustrates the complexity of the evolution and highlights the difficulty in predicting precise molecular function in this subfamily. However, additional data improves predictive capabilities in a protein family such as the Iα aminotransferases where substrate specificity changes occur repeatedly in the family's evolution. Additionally, further studies that build on the sequence, phylogenetic, and kinetic data presented here can be targeted to identify the cellular function of Iα aminotransferases as well as the role of particular conserved or variable residues in the subfamily. In particular, although our analysis indicates that the active sites of the plant enzymes are relatively conserved, the overall sequences are distinct from the other enzymes for which we now have activity data. We have shown here that closely related enzymes often have different specificities and the extent of such diversity in the other regions of the phylogeny remains an area for future study. Together with the continued deposition of aminotransferase crystal structures, the kinetics data presented here rejuvenates this subfamily for new insights into sequence-structure-function relationships.

REFERENCES

1. Gelfand DH, Steinberg RA. Escherichia coli mutants deficient in the aspartate and aromatic amino acid aminotransferases. J Bacteriol 1977;130:429–440.
2. Gu W, Song J, Bonner CA, Xie G, Jensen RA. PhipC is an essential aminotransferase for aromatic amino acid catabolism in Pseudomonas aeruginosa. Microbiology 1998;144:3127–3134.
3. Finn RD, Mistry J, Tate J, Coggill P, Heger A, Pollington JE, Gavin NJ, Ceric G, Forslund K, Holm L, Sonhammer EL, Eddy SR, Bateman A. The Pfam protein families database. Nucleic Acids Res 2010;38(Database issue):D212–D222.
4. Mehta PK, Hale TL, Christen P. Aminotransferases: demonstration of homology and division into evolutionary subgroups. Eur J Biochem 1993;214:549–561.
5. Jensen RA, Gu W. Evolutionary recruitment of biochemically specialized subdivisions of Family I within the protein superfamily of aminotransferases. J Bacteriol 1996;178:2161–2171.
6. Sivaraman S, Kirsch JE. The narrow substrate specificity of human tyrosine aminotransferase—the enzyme deficient in tyrosinemia type II. FEBS J 2006;273:1920–1929.
7. Nowicki C, Hunter GR, Montemartini-Kalisz M, Blankenfeldt W, Hecht H, Kaisler HM. Recombinant tyrosine aminotransferase from Trypanosoma cruzi: structural characterization and site directed mutagenesis of a broad substrate specificity enzyme. Biochim Biophys Acta 2001;1546:268–281.
8. Brenner SE. Errors in genome annotation. Trends Genet 1999;15:132–133.
9. Devos D, Valencia A. Intrinsic errors in genome annotation. Trends Genet 2001;17:429–431.
10. Schnoes AM, Brown SD, Dodevski I, Babbitt PC. Annotation error in public databases: misannotation of molecular function in enzyme superfamilies. PLoS Comput Biol 2009;5:e1000605.
11. Dean CR, Franklund CV, Retief JD, Cosyne J, M., Hatano K, Evans DJ, Pier GB, Goldberg JB. Characterization of the serogroup O11 O-antigen locus of Pseudomonas aeruginosa PA103. J Bacteriol 1999;181:4275–4284.
12. Zhao G, Xia T, Song J, Jensen RA. Pseudomonas aeruginosa possesses homologues of mammalian phenylalanine hydroxylase and 4 alpha-carbinolamine dehydratase/DCHD as part of a three-component gene cluster. Proc Natl Acad Sci USA 1994;91:1366–1370.
13. DeLano WL. The PyMOL molecular graphics system. 0.99. San Carlos, CA: DeLano Scientific; 2002.
14. Ford GC, Eichele G, Jansonius JN. Three-dimensional structure of a pyridoxal-phosphate-dependent enzyme, mitochondrial aspartate aminotransferase. Proc Natl Acad Sci USA 1980;77:2539–2563.
15. Jeffery CJ, Barry T, Doonan S, Petsko GA, Ringe D. Crystal structure of Saccharomyces cerevisiae cytosolic aspartic aminotransferase. Protein Sci 1998;7:1380–1387.
16. Kamitori S, Okamoto A, Hirotsu K, Higuchi T, Kuramitsu S, Kagamiyama H, Matsuura Y, Katsube Y. Three-dimensional structures of aspartate aminotransferase from Escherichia coli and its mutant enzyme at 2.5 Å resolution. J Biochem (Tokyo) 1990;108:175–184.
17. Malashkevich VN, Strokovropov BV, Borisov VV, Dauter Z, Wilson KS, Torchinsky YM. Crystal structure of the closed form of chicken cytosolic aspartate aminotransferase at 1.9 Å resolution. J Mol Biol 1995;247:111–124.
18. Okamoto A, Nakai Y, Hayashi H, Hirotsu K, Kagamiyama H, Crystal structures of Plasmodium falciparum aromatic amino acid aminotransferase: a substrate recognition site constructed by rearrangement of hydrogen bond network. J Mol Biol 1998;280:443–461.
19. Rhee S, Silva MM, Hyde CC, Rogers PH, Metzler CM, Metzler DE, Arnone A. Refinement and comparisons of the crystal structures of pig cytosolic aspartate aminotransferase and its complex with 2-methylnapata. J Biol Chem 1997;272:17293–17302.
20. Han Q, Cai T, Tagle D, Li J. Structure, expression, and function of kynurenine aminotransferases in human and rodent brains. Cell Mol Life Sci 2010;67:353–368.
21. Wrenger C, Muller IB, Schifferdecker AJ, Jain R, Jordanova R, Groves MR. Specific inhibition of the aspartate aminotransferase of Escherichia coli. J Mol Biol 2011;405:956–971.
22. Wilson CA, Kreychman J, Gerstein M. Assessing annotation transfer for genomics: quantifying the relations between protein sequence, structure and function through traditional and probabilistic scores. J Mol Biol 2000;297:233–249.
23. Luong TN, Kirsch JF. A general method for the quantitative analysis of functional chimeras: applications from site-directed mutagenesis and macromolecular association. Protein Sci 2001;10:581–591.
24. Goss LM, Planas A, Kirsch JF. Contribution to catalysis and stability of the five cysteines in Escherichia coli aspartate aminotransferase. Preparation and properties of a cysteine-free enzyme. Biochemistry 1992;31:32–39.
25. Hayashi H, Inoue K, Nagata T, Kuramitsu S, Kagamiyama H. Escherichia coli aromatic amino acid aminotransferase: characterization and comparison with aspartate aminotransferase. Biochemistry 1993;32:12229–12239.
26. Onuffer JJ, Kirsch JF. Redesign of the substrate specificity of Escherichia coli aspartate aminotransferase to that of Escherichia coli tyrosine aminotransferase by homology modeling and site-directed mutagenesis. Protein Sci 1995;4:1750–1757.
27. Shaffer WA, Luong TN, Rothman SC, Kirsch JF. Quantitative chimeric analysis of six specificity determinants that differentiate Escherichia coli aspartate from tyrosine aminotransferase. Protein Sci 2002;11:2848–2859.
28. Rothman SC, Kirsch JF. How does an enzyme evolved in vitro compare to naturally occurring homologs possessing the targeted function? Tyrosine aminotransferase from aspartate aminotransferase. J Mol Biol 2003;327:593–608.
29. Onuffer JJ, Kirsch JF. Characterization of the apparent negative cooperativity induced in Escherichia coli aspartate aminotransferase by
the replacement of Asp222 with alanine. Evidence for an extremely slow conformational change. Protein Eng 1994;7:413–424.

30. Aitken SM, Kim DH, Kirsch JF. Escherichia coli cystathionine gamma-synthase does not obey ping-pong kinetics. Novel continuous assays for the elimination and substitution reactions. Biochemistry 2003;42:11297–11306.

31. Muratore KE, Srouji JR, Chow MA, Kirsch JF. Recombinant expression of twelve evolutionarily diverse subfamily I[alpha] aminotransferases. Protein Expr Purif 2008;57:34–44.

32. Karmen A, Wroblewski F, Ladue JS. Transaminase activity in human blood. J Clin Invest 1955;34:126–133.

33. Luong TN, Kirsch JF. A continuous coupled spectrophotometric assay for tyrosine aminotransferase activity with aromatic and other nonpolar amino acids. Anal Biochem 1997;253:46–49.

34. Velick SF, Vavra J. A kinetic and equilibrium analysis of the glutamic oxaloacetate transaminase mechanism. J Biol Chem 1962;237:2109–2122.

35. Boeckmann B, Bairoch A, Apweiler R, Blatter M-C, Estreicher A, Gasteiger E, Martin MJ, Michoud K, O’Donovan C, Phan I, Pilbout S, Schneider M. The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003. Nucleic Acids Res 2003;31:365–370.

36. Edgar RC. SATCHMO: sequence alignment and tree construction using hidden Markov models. Bioinformatics 2003;19:1404–1411.

37. Zhang Z, Lindstam M, Urge P, Peterson C, Lu G. Potential for Dramatic Improvement in Sequence Alignment against Structures of Remote Homologous Proteins by Extracting Structural Information from Multiple Structure Alignment. J Mol Biol 2003;332:2127–32142.

38. Boirech A, Apweiler R, Wu CH, Barker WC, Boeckmann B, Ferro S, Gasteiger E, Huang L, Lopez R, Magrane M, Martin NI, Natale DA, O’Donovan C, Redaschi N, Ye P, S. The Universal Protein Resource (UniProt). Nucleic Acids Res 2005;33(Suppl 1):D154–D159.

39. Cronin VB, Maras B, Barra D, Doonan S. The amino acid sequence of the aminotransferase from baker’s yeast (Saccharomyces cerevisiae). Biochem J 1991;277:355–340.

40. Engelhardt BE, Jordan MI, Muratore KE, Brenner SE. Protein molecular function prediction by bayesian phylogenomics. PLoS Comput Biol 2005;1:e45.

41. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 2004;32:1792–1797.

42. Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 2006;22:2688–2690.

43. Stamatakis A, Hoover P, Rougemont J. A rapid bootstrap algorithm for the RAXML Web servers. Syst Biol 2008;57:758–771.

44. Felsenstein J. An alternating least squares approach to inferring phylogenies from pairwise distances. Syst Biol 1997;46:101–111.

45. Engelhardt BE, Jordan MI, Srouji JR, Brenner SE. Genome-scale phylogenetic function annotation of large and diverse protein families. Genome Res 2011;21:1969–1980.

46. Miyazawa K, Kawaguchi S, Okamoto A, Kato R, Ogawa T, Kuramitsu S. Construction of aminotransferase chimeras and analysis of their substrate specificity. J Biochem (Tokyo) 1994;115:568–577.

47. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997;25:4876–4882.

48. Iano T, Ou S, Kagamiyama H. Directed evolution of an aspartate aminotransferase with new substrate specificities. Proc Natl Acad Sci USA 1998;95:5511–5515.

49. Brooks DJ, Fresco JR. Increased frequency of cysteine, tyrosine, and phenylalanine residues since the last universal ancestor. Mol Cell Proteomics 2002;1:125–131.

50. Jansonius JN, Eichele G, Ford GC, Picot D, Thaller C, Vincent MG. Spatial structure of mitochondrial aspartate aminotransferase. In: Christen P, Metzler DE, editors. Transaminases. Vol. 2. Biochemistry. New York: John Wiley & Sons, Inc.; 1985. pp 110–137.

51. Sandmeier E, Christen P. Chemical modification of a functional arginyl residue (Arg 292) of mitochondrial aspartate aminotransferase. Identification as the binding site for the distal carboxylate group of the substrate. J Biol Chem 1982;257:6745–6750.

52. Slebe JC, Martinez-Carrion M. Carbamylation of aspartate transaminase and the pK value of the active site lysyl residue. J Biol Chem 1976;251:5663–5669.

53. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 2000;25:25–29.

54. Engelhardt BE, Stephens M. Analysis of population structure: a unifying framework and novel methods based on sparse factor analysis. PLoS Genet 2010;6:e1001117.

55. Oue S, Okamoto A, Nakai Y, Nakahira M, Shibatani T, Hayashi H, Kagamiyama H. Paracoccus denitrificans aromatic amino acid aminotransferase: a model enzyme for the study of dual substrate recognition mechanism. J Biochem (Tokyo) 1997;121:161–171.

56. Pan QW, Tanase S, Fukumoto Y, Nagashima F, Rhee S, Rogers PH, Arnone A, Morino Y. Functional roles of valine 37 and glycine 38 in the mobile loop of porcine cytosolic aspartate aminotransferase. J Biol Chem 1993;268:24758–24765.

57. Pan P, Iaissi R, Gehring H, Giannattasio S, Christen P. Shift in pH-rate profile and enhanced discrimination between dicarboxylic and aromatic substrates in mitochondrial aspartate aminotransferase Y70H. Biochemistry 1994;33:2757–2760.

58. Lowry OH, Carter J, Ward JB, Glaser L. The effect of carbon and nitrogen sources on the level of metabolic intermediates in Escherichia coli. J Biol Chem 1971;246:6511–6521.

59. Berger LC, Wilson J, Wood P, Berger BJ. Methionine regeneration and aspartate aminotransferase in parasitic protozoa. J Bacteriol 2001;183(15):4421–4434.

60. Fukumoto Y, Tanase S, Nagashima F, Ueda S, Ikegami K, Morino Y. Structural and functional role of the amino-terminal region of porcine cytosolic aspartate aminotransferase. Catalytic and structural properties of enzyme derivatives truncated on the amino-terminal side. J Biol Chem 1991;266:4187–4193.

61. Vernal J, Cazzulo JJ, Nowicki C. Isolation and partial characterization of a broad specificity aminotransferase from Leishmania mexicana promastigotes. Mol Biochem Parasitol 1998;96:83–92.

62. Morin PJ, Subramanian GS, Gilmore TD. AAT1, a gene encoding a mitochondrial aspartate aminotransferase in Saccharomyces cerevisiae. Biochem J 1995;321(2):111–116.

63. Shulitz CJ, Coruzzi GM. The aspartate aminotransferase gene family of Arabidopsis encodes isozymes localized to three distinct subcellular compartments. Plant J 1995;7:61–75.

64. Young ET, Pilgrim D. Isolation and DNA sequence of ADH3, a nuclear gene encoding the mitochondrial isozyme of alcohol dehydrogenase in Saccharomyces cerevisiae. Mol Cell Biol 1985;5:3024–3034.

65. Wilkie SE, Warren MJ. Recombinant expression, purification, and characterization of three isozymes of aspartate aminotransferase from Arabidopsis thaliana. Protein Expr Purif 1998;12:381–389.

66. Eisen JA. Phylogenomics: improving functional predictions for uncharacterized genes by evolutionary analysis. Genome Res 1998;8:163–167.