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Recombinant expression of twelve evolutionarily diverse subfamily Iα aminotransferases

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Abstract

Aminotransferases are essential enzymes involved in the central metabolism of all organisms. The Iα subfamily of aspartate and tyrosine aminotransferases (AATases and TATases) is the best-characterized grouping, but only eight enzymes from this subfamily, representing relatively little sequence diversity, have been experimentally characterized for substrate specificity (*i.e.*, AATase *vs.* TATase). Genome annotation, based on this limited dataset, provides tentative assignments for all sequenced members of this subfamily. This procedure is, however, subject to error, particularly when the experimental basis set is limited. To address this problem we cloned twelve additional subfamily Iα enzymes from an evolutionarily divergent set of organisms. Nine were purified to homogeneity after heterologous expression in *Escherichia coli* in native, intein-tagged or His_6-tagged forms. The two *Saccharomyces cerevisiae* isoforms were recombinantly produced in yeast. The effects of the C-terminal tags on expression, purification and enzyme activity are discussed.

Keywords: Aminotransferase; Pyridoxal phosphate; Intein; Heterologous expression; Substrate specificity; Annotation

Aminotransferases are pivotal enzymes in cellular metabolism and are the core members of the family Iα aminotransferases [1,2]. They are pyridoxal 5′-phosphate dependent (PLP[3]) enzymes that catalyze the reaction shown in Scheme 1.

Aspartate aminotransferases (AATases) are specific for the dicarboxylic amino acids, L-glutamate and L-aspartate, and their corresponding α-keto acids, α-ketoglutarate (αKG) and oxaloacetate. These enzymes function in linking carbohydrate to amino acid metabolism, and are ubiquitous. They serve additionally in the eukaryotic malate shuttle to translocate hydride equivalents between the cytosol and mitochondria. AATases are constitutive in *Escherichia coli* [3,4]. Tyrosine aminotransferases (TATases) play a variety of roles, the most prominent of which are the biosynthesis and catabolism of phenylalanine and tyrosine [5–7].

Given their importance, it is somewhat surprising that the substrate specificities of only eight aminotransferases have been fully characterized [6,8–13]. Many more have had specificity assigned by genomic annotation, but, error rates are generally estimated to be *ca.* 33% for substrate specificity assignments [14]. As with other protein families, aminotransferase annotations are often assigned based on sequence similarity. Of the eight aminotransferases with experimentally determined substrate specificities, three are from chicken or pig, two are the *E. coli* orthologs, and the remaining three are from other proteobacteria. To date, all subfamily Iα TATases have been isolated from bacteria,

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[3] *Abbreviations used:* AATase, aspartate aminotransferase; AtcAT, AtmAT, CecAT, CtAT, GicAT, PaAT, PfcAT, SccAT, ScmAT, TbcAT, TbmAT, and VcAT, aminotransferases (see Table 1 for sources); CBD, chitin binding domain; HA, hemagglutination; MCS, multiple cloning site; MDH, malate dehydrogenase; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; PMSF, phenylmethanesulfonyl fluoride; TATase, tyrosine aminotransferase; αKG, α-ketoglutarate.

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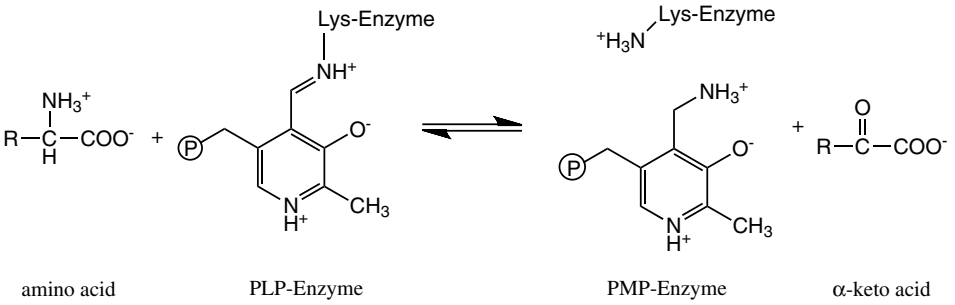

Scheme 1. General aminotransferase reaction.

while AATases have been found in both eukaryotes and bacteria. Given this limited set of experimental data and the errors associated with propagation of genome annotation, it is not surprising that the majority of enzymes in this family have been assigned as AATases.

Alternative methods of substrate specificity assignment have been developed that do not rely on overall sequence similarity (*e.g.*, SIFTER [15]), but aminotransferases have proven to be particularly challenging due to multiple, independent specificity swapping points throughout evolutionary history of this family (Barbara Engelhardt and Michael Jordan, personal communication). Further, there is experimental evidence from both rational redesign and directed evolution for alternate specificity determinants [16–18]. The limited number of AAT/TATases with biochemically determined specificity, taken together with the importance of these enzymes in investigations of rationally designed [19,20] and directed evolution reversal of specificity [21,22], provide a strong motivation for characterization of a larger and more diverse variety of AATases and TATases. We report here the purification and initial kinetic investigations of eleven additional members of this group.

Materials and methods

All cloning enzymes were obtained from New England Biolabs (Ipswich, MA) except that alkaline phosphatase was from USB (Cleveland, OH), Pfu polymerase was from Stratagene (La Jolla, CA), AarI was from Fermentas (Burlington, Ontario, Canada), and all GATEWAY cloning reagents, including enzymes, were from Invitrogen (Carlsbad, CA). Purification of DNA fragments was carried out using GFX kits from GE Healthcare (Piscataway, NJ). DNA plasmid purification was done with Wizard Miniprep or Midiprep kits from Promega (Madison, WI). All cloning products were confirmed by DNA sequencing performed by Elim Biopharmaceuticals (Hayward, CA).

Hydroxyapatite resin was a Bio-Rad product (Hercules, CA). All pre-packed chromatography columns and Sephadex G-75 and G-100 were purchased from GE Healthcare. NiNTA resin was from GE Healthcare or Qiagen (Valencia, CA). Research Products International (Prospect, IL) was the supplier for IPTG and DTT. SDS–PAGE protein bands were stained with GelCode Blue (Hercules, CA) or Blue BandIt (Solon, OH). All other reagents were from Sigma–Aldrich (St. Louis, MO) or Fisher (Fairlawn, NJ). Protease inhibitor mixtures added to cell lysates included 10 mM EDTA, 50 μM phenylmethanesulfonyl fluoride (PMSF), 1 μg/mL E-64 and 1 μg/mL pepstatin A. Malate dehydrogenase (MDH) was prepared as previously described [20]. Mouse α-His and HRP-conjugated goat α-mouse antibodies (from Qiagen and GE Healthcare, respectively) were used for western blot analyses.

Plasmids and strains

pUC18 was obtained from New England Biolabs, pET-19b and pET-43.1a from EMD (San Diego, CA), and pDONR207 from Invitrogen. pTYB1 is part of the IMPACT-CN system from New England Biolabs (Ipswich, MA). pTYB1-4 was generated by adapter cloning of pTYB1. Briefly, pTYB1 was sequentially digested with EcoRI and SapI and ligated to a pair of sticky-end DNA oligomers (5′-AATTCCTCGCACCTGCTTCC-3′ and 5′-GCAGGAAGCAGGTGCGAGG-3′). Four-base overhangs that are complementary to AarI-digested pTYB1-4 can be generated with about a dozen restriction enzymes (Fig. 1). Chaperone co-expression plasmids, including pKJE7 and pGro7, were from Takara Bioscience (Shiga, Japan). pLysSRare was isolated from *E. coli* Rosetta (DE3)pLysS cells from EMD. pVV214 [23] was a gift from Jasper Rine (University of California, Berkeley).

Plasmid or genomic DNA containing aminotransferase genes were from the sources listed in Table 1. *E. coli* strain DH10B is from Invitrogen and strain MG204 (*his-23(am) proB trpA-605(am) lacI3 lacZ118(oc) gyrA rpsL aspC::kanR tyrB RecA::Tn10 ilvE*) was a gift from Ian Fotheringham (Nutrasweet, Mount Prospect, IL). MG204(DE3) was created by lysogenizing MG204 with λDE3 using the Novagen λDE3 lysogenization kit from EMD. *E. coli* strains were plasmid transformed by electroporation with a Bio-Rad GenePulser or by heat shock. *Saccharomyces cerevisiae* strain BCY123 (*MATa*, *Can1*, *ade2*, *trp1*, *Ura3-52*, *his3*, *leu2-3*, *112*, *pep4*::*his*$^+$, *prb1*::*leu2*$^+$, *bar1*::*HisG*$^+$, *lys2*::*pGAL1/10-GAL4*$^+$) was a gift from James Berger (University of California, Berkeley). *S. cerevisiae* was plasmid transformed by heat shock.

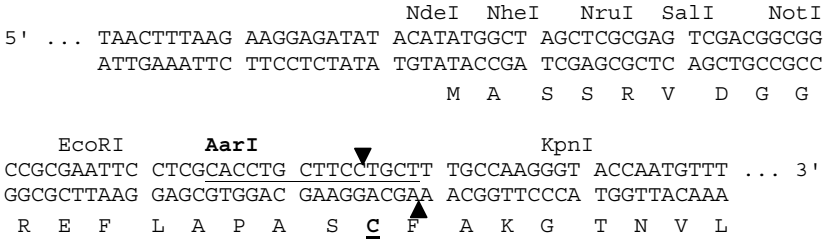

Fig. 1. Multiple cloning site (MCS) of pTYB1-4. pTYB1-4 was created from pTYB1 as described in Materials and methods. The MCS of pTYB1-4 contains a restriction site for AarI (underlined), which yields a 4-base 3′-overhang (indicated by arrows) in contrast to the 3-base overhang remaining after digestion of pTYB1 with SapI. Unchanged restriction sites are noted in normal font, and the new AarI site is in bold. XhoI and SapI restriction sites are absent in the pTYB1-4 MCS. The one-letter amino acid translation is shown with the intein cysteine in bold, underlined font.

Table 1
Sources of aminotransferase genes

	Organism (organelle)	SwissProt ID	Source
AtcAT	*Arabidopsis thaliana* (cytosol)	AAT4_ARATH	Plasmid Gift from Gloria Coruzzi[a]
AtmAT	*Arabidopsis thaliana* (mitochondria)	AAT1_ARATH	Plasmid Gift from Gloria Coruzzi[a]
CecAT	*Caenorhabditis elegans* (cytosol)	AATC_CAEEL	Plasmid Open Biosystems (Clone g_yk4377)
CtAT	*Chlamydia trachomatis*	O84642	gDNA (strain L2) Gift from Richard Stephens[b]
GicAT	*Giardia intestinalis* (cytosol)	Q964E9	Plasmid Gift from Bradley Berger[c]
PfcAT	*Plasmodium falciparum* (cytosol)	O96142	Plasmid Gift from Bradley Berger[c]
PaAT	*Pseudomonas aeruginosa*	AAT_PSEAE	gDNA (strain PA01) Gift from Steven Lindow (cells)
SccAT	*Saccharomyces cerevisiae* (cytosol)	AATC_YEAST	Plasmid Open Biosystems (Clone YLR027C)
ScmAT	*Saccharomyces cerevisiae* (mitochondria)	AATM_YEAST	Plasmid Gift from Tom Gilmore[d]
TbcAT	*Trypanosoma brucei* (cytosol)	Q964F1	Plasmid Gift from Bradley Berger[c]
TbmAT	*Trypanosoma brucei* (mitochondria)	Q964F0	Plasmid Gift from Bradley Berger[c]
VcAT	*Vibrio cholerae*	Q9KM75	gDNA (strain N16961) Gift from Matthew Waldor[e]

[a] New York University [37].
[b] University of California, Berkeley.
[c] Defense Research and Development Canada [38].
[d] Boston University [39].
[e] Purdue University.

General cloning procedures for aminotransferases

A yeast expression vector with the *S. cerevisiae* cytosolic aminotransferase (SccAT) gene from Open Biosystems (Huntsville, AL) was used for expression of SccAT. All other aminotransferase genes were cloned by PCR using Pfu polymerase (except as noted below) using the annealing temperature listed in Table 2. The resulting fragments contained a 5′ NdeI site and the appropriate 3′ restriction site. PCR primers and the 3′ endonuclease used in cloning are given in Table 2. The digested PCR fragments were purified from agarose gels with GFX spin columns and ligated to pTYB1 or pTYB1-4 that were similarly digested, dephosphorylated with shrimp alkaline phosphatase, and also gel-purified.

These genes were subcloned from pTYB1 or pTYB1-4 into pET-43.1a to change the fused affinity tag. In most cases, the pTYB1-derived vectors were digested with NdeI and KpnI. The resulting genes have the first 4 codons from the pTYB1 intein tag (coding Cys-Phe-Ala-Lys) and 38

Table 2
Primers used for PCR of aminotransferase genes

Gene	Name	Primer sequence	T_{anneal} (°C)	Plasmid	pTYB 3′ endonuclease
AtcAT	Atc	5′-CCAATTCATATGAATTCCATCTTGTCAAGCGTCCTTCC-3′			
	rAtc	5′-CCAGAACACCTGCTTTAAGCAGGCGATG-3′	45	pTYB1-4	AarI
	B1Atc	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTACATGAATTCCATC TTGTCA-3′			
	B2Atc	5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGTGGTGATGGTG ATGGTGATGATGGGCGATGCGAGTAACAAC-3′	60	pENTR207	
AtmAT	Atm	5′-CATAGACATATGGCTTTGGCGATGATGATCCG-3′			
	rAtm	5′-CACATGCACCTGCATGAAGCAAGATGACTTG-3′	57	pTYB1-4	AarI
CecAT	Cec	5′-CCAAGCACATATGTCCTTCTTTGACGGAATCCC-3′			
	rCec	5′-CTTGTACACCTGCGCTGAGCAGATGTTGCTC-3′	60	pTYB1-4	AarI
CtAT	Ct	5′-CGAGAGGTTTTCATATGAGTCTTTTTGAACAGTTACCTTCC-3′			
	rCt	5′-CTGAACGAAGACCGAGCATGATACGGACC-3′	55	pTYB1-4	BbsI
	CtQC	5′-TTTGACGATGAGAAAGATAAAAATTATCTACCCATCAAAGGATCGTC-3′			
	rCtQC	5′-GACGATCCTTTGATGGGTAGATAATTTTTATCTTTCTCATCGTCAAA-3′	68		
GicAT	Gic	5′-CACGACCATATGTCTGTCTTCTCAG-3′			
	rGic	5′-CCAAGCTCTTCCGCATTTCTTGAA-3′	55	pTYB1	SapI
PaAT	PaA	5′-CGCGAATTCATGAGTCTGTTTTCTGCCGTCGAAATG-3′			
	rPaA	5′-CGCGGATCCCTACAGGACCTGGACGATGGCCTTGGT-3′′	55	pKK223-3	
	PaB	5′-AATTCATGAGTCTGTTTTCTGCCGTCGAAATGGCACCGC-3′			
	rPaB	5′-CGGGATCCTTACAGGACCTGGACAATTGCCTTGGTG-3′	60	pUC18	
	rPaBH	5′-CGGGATCCTTAGTGGTGGTGGTGGTGATGCAGGACCTGTACAATTG CCTTGGTGATGG-3′			
PfcAT	Pfc	5′-CACGACCATATGGATAAGTTATTAAGCAGC-3′			
	rPfc	5′-CGGAACGAAGACCGAGCATATTTG-3′	45	pTYB1-4	BbsI
ScmAT	Scm	5′-CAAGACCATATGTTGCGGACGCGGCTTA-3′			
	rScm	5′-CTGAACTGTAAGGAAGACAAAGCATGCGAGTTTGTC-3′	55	pTYB1-4	BbsI
	B1Scm	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTACATGACGTCATCG CTTAGT-3′			
	B2Scm	5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGTGGTGATG GTGATGGTGATGATGGA GTTTGTCCATTTT-3′	60	pENTR207	
TbcAT	Tbc	5′-CGCCATATGTCCCGCCCGTTTAAGG-3′			
	rTbc	5′-CGCGCTCTTCCGCACTTGTTACGCACGTGTCGG-3′	58	pTYB1	SapI
TbmAT	Tbm	5′-CGAAATCATATGGGGAAACCGGACC-3′			
	rTbm	5′-CGGAACGAAGACCGAGCATTTAGTAAC-3′	50	pTYB1-4	BbsI
VcAT	Vc	5′-CTTTGTCATATGTTACCTGAATTTCAACGGGTTGTGACTTT-3′			
	rVc	5′-CGCTGTCGCTCTTCTGCACGACACA-3′	55	pTYB1	SapI

codons from pET-43.1a, including those for the 6 C-terminal histidines (Fig. 2). *E. coli* DH10B was used for all cloning steps. These cloning procedures were significantly altered in five cases as detailed below.

Cloning Arabidopsis thaliana cytosolic aminotransferase (AtcAT) for expression in E. coli and S. cerevisiae

Two rounds of PCR with the Atc and rAtc primers (Table 2) were performed in the cloning of AtcAT. After the first, bands were visible on an agarose gel corresponding to the expected 1.2 kb product as well as undesired 2 and 4 kb fragments (*ca* 1:1:2 mixture in order of increasing size). The gel-purified 1.2 kb fragment served as the template for the second PCR reaction. This PCR product was digested with NdeI and AarI restriction endonucleases, and ligated to NdeI/AarI-digested pTYB1-4.

The gene bears an internal KpnI site, which is identical to that required for subcloning into pET-43.1a. Therefore the AtcAT-containing vector was fully digested with an excess of NdeI, then partially digested with KpnI. Finally, the ~1000 base pair insert was gel-purified and ligated to NdeI/KpnI digested pET-43.1a.

Heterologous expression in *S. cerevisiae* was attempted from a pVV214-based vector. To make this construct, PCR amplification of the gene from the original AtcAT plasmid was done using a primer containing the attb1 recombination site and a primer containing eight His codons and the attb2 recombination site (B1Atc and B2Atc in Table 2, respectively). It was necessary to do the first ten cycles of the PCR reaction with a 40 °C anneal-

```
                    KpnI                    HindIII EagI
5' ... TGCTTTGCCA AGGGTACCAT CGATACGCGT TCGAAGCTTG CGGCCGCACA
       C  F  A  K   G  T  I   D  T  R   S  K  L  A   A  A  Q

              PmlI
       GCTGTATACA CGTGCAAGCC AGCCAGAACT CGCTCCTGAA GACCCAGAGG
        L  Y  T   R  A  S  Q   P  E  L   A  P  E   D  P  E  D

          XhoI
       ATCTCGAGCA CCACCACCAC CACCACTAAT GTTAATTAAG ... 3'
         L  E  H   H  H  H   H  H  *
```

Fig. 2. Sequence of pET-43.1a aminotransferase constructs. Aminotransferases were subcloned from the pTYB-based constructs into pET-43.1a(+) with the KpnI endonuclease (for 3′ sticky end). Consequently, bases from both the pTYB1 and pET-43.1a vectors were fused to the 3′ end of the aminotransferase gene, coding for 42 additional amino acids including the His_6 affinity tag.

ing temperature before increasing to 60 °C for the last twenty. Invitrogen's GATEWAY cloning protocol was followed to generate pENTR207-AtcAT by recombination of the PCR product with pDONR207. pENTR207-AtcAT was then recombined with pVV214 to generate pEXPVV214-AtcAT.

Cloning Chlamydia trachomatis aminotransferase (CtAT) from genomic DNA for E. coli expression

This gene was cloned from genomic DNA using Taq polymerase with the addition of 4.5 mM $MgCl_2$. BbsI was used as the 3′ restriction endonuclease, which yielded a four-base overhang that could be ligated to AarI-digested pTYB1-4. Multiple clones were sequenced to confirm the wild-type sequence[4]. QuikChange Mutagenesis was performed to revert a premature stop (TAA) at codon 67 to the wild-type tyrosine codon (TAT) in the best clone. Briefly, after PCR amplification of the entire plasmid with the primers CtQC and rCtQC, the parent product with the premature stop codon was digested with DpnI before the purified PCR products were transformed in DH10B. The resulting sequence has the start codon intentionally mutated from GTG to ATG and an additional silent serine mutation at codon 351 from wild-type TCA to TCG.

Cloning Pseudomonas aeruginosa aminotransferase (PaAT) from genomic DNA for native and affinity-tagged E. coli expression

This gene was cloned from genomic DNA purified from *P. aeruginosa* strain PA01. 10% Glycerol was required in the PCR reaction mixture. The PCR product that was generated using the PaA and rPaA primers and pKK223-3 were sequentially digested with BamHI and EcoRI, and ligated to produce pKMK4. In order to improve expression levels, the gene was subcloned into other vectors including pUC18 and pET-19b. Silent restriction sites were mutated into the PaAT gene by PCR of pKMK4 using the primers PaB and rPaB (for native) or rPaBH (for His_6-tagged). The PCR fragment and pUC18 were sequentially digested with EcoRI and BamHI and ligated to yield pKMU1 (and pKMU1H for the His_6-tagged variant). In order to place the gene into pET-19b, the 5′ restriction site needed to be specific for NcoI and not EcoRI, therefore adapters with the sequences, 5′-CATGAGTCTGTTTTCT GCCGTCGAAATGGCACCGC-3′ and 5′-GTGCCATT TCGACGGCAGAAAACAGACT-3′, were ligated to DraIII digested pKMU1. The resulting ligation product was digested with BamHI and ligated to NcoI/BamHI digested pET-19b (to make pKME1 and pKME1H).

Cloning S. cerevisiae mitochondrial aminotransferase (ScmAT) for recombinant expression in E. coli and S. cerevisiae

As this gene has an internal KpnI site, subcloning into pET-43.1a was accomplished by the method described above for AtcAT. The mitochondrial signal sequence is predicted by TargetP [24,25] to be encoded by the first sixteen amino acids of the ScmAT gene. Consequently, for yeast expression, the gene was PCR amplified from the original plasmid with a 5′ primer starting at codon 17 preceded by a Met codon in addition to an upstream attb1 recombination site (B1Scm), while the 3′ primer contained an attb2 recombination site preceded by eight His codons (B2Scm). PCR amplification and GATEWAY cloning was done as described above for AtcAT such that the ScmAT PCR product was recombined with pDONR207 to generate pENTR207-ScmAT, which was then recombined with pVV214 to give pEXPVV214-ScmAT.

Cloning Vibrio cholerae aminotransferase (VcAT) from genomic DNA for E. coli expression

VcAT was cloned from genomic DNA using Taq polymerase, as was employed for CtAT cloning, except that the reaction contained 4 mM $MgCl_2$. Most sequenced clones contained some mutations and, because of an error in the genome database for the 5′ sequence of VcAT, the clones that we isolated have lost the intended NdeI restriction site. Instead they have 19 nucleotides cloned from the upstream portion of the genome inserted in front of the

[4] Genbank Accession No. AY937244.

start codon. While there were point mutations in some clones, we were able to isolate one with none. That gene was subcloned into pET-43.1a using the XbaI and KpnI restriction sites, as the NdeI site was lost in the pTYB1 cloning process.

Expression

The aminotransferases that were cloned into pET or pTYB vectors were expressed in strain MG204(DE3) in 2YT rich media in the presence of 0.1% pyridoxine and selective antibiotics. They were induced with 0.3 mM IPTG and grown at 30 °C for 3–6 h or overnight for the pET and pTYB constructs, respectively; except that His_6-tagged PaAT, *A. thaliana* mitochondrial (AtmAT), *Caenorhabditis elegans* cytosolic (CecAT), and *T. brucei* cytosolic (TbcAT) enzymes were expressed for 3–6 h at 37 °C. PaAT was similarly expressed from pKMK4 for 6 h at 30 °C, but the expression strain used was MG204 (*i.e.*, not lysogenized). Intein-tagged *P. falciparum* cytosolic aminotransferase (PfcAT) was successfully co-expressed with chaperones from pKJE7 (DnaK/DnaJ/GrpE) or rare tRNAs from pLysSRare, although these helper plasmids were unnecessary for His_6-tagged PfcAT. GroES/L co-expression from pGro7 was essential for soluble expression of His_6-tagged *T. brucei* mitochondrial enzyme (TbmAT). Cells were lysed by sonication (except that untagged PaAT was lysed with a French press) in a buffer appropriate for the purification method containing protease inhibitors.

Aminotransferase genes in yeast vectors were transformed into *S. cerevisiae* BCY123 and grown on SD-ura plates. After liquid culture growth at 30 °C in S-ura + 2% raffinose to an A_{600} of 0.5, expression was induced by growth in YP + 2% galactose. Cells were harvested after overnight growth by centrifugation and lysed with a mortar and pestle under liquid nitrogen.

Purification of aminotransferases

Cell lysates were clarified by centrifugation and all purification steps were carried out at 4 °C in buffers containing 10 μM PLP. Aminotransferase-containing fractions were identified by MDH-coupled spectrophotometric assays [26]. The solutions contained 200 mM TAPS, pH 8.0, 100 mM KCl, 150 μM NADH, 10 μM PLP, 5 mM L-aspartate and 5 mM αKG. Product formation was measured by decrease in A_{340} due to NADH oxidation. Protein purity was determined by SDS–PAGE. Purified fractions containing AATase activity were pooled and dialyzed against 20 mM potassium phosphate pH 8.0 with 10 μM PLP to remove sodium ions or imidazole. The enzymes were stored in 40–50% glycerol at −20 °C. Protein concentration was calculated from the A_{280} using extinction coefficients predicted by ProtParam [27].

Proteins expressed from pTYB1-based vectors have C-terminal intein and chitin-binding domains. They were purified over chitin resin (New England Biolabs) in buffer containing 20 mM sodium phosphate, pH 8.0 with 500 mM NaCl and 10 μM PLP. The resin-bound protein was incubated overnight in the same buffer with 50 mM DTT to catalyze cleavage of the tag, and eluted with 20 mM potassium phosphate buffer containing 10 μM PLP.

C-terminal His_6-tagged proteins expressed from the pET-43.1a constructs were purified with either NiNTA FastFlow (GE Healthcare) or NiNTA Superflow (Qiagen) resin at pH 8.0. The loading buffer was 20 mM sodium phosphate, pH 8.0, 500 mM NaCl, 20 mM imidazole and 10 μM PLP. The proteins were eluted with a linear or multi-step gradient to 20 mM potassium phosphate, pH 8.0, 500 mM imidazole and 10 μM PLP. PaAT expressed from pKME1H and the proteins recombinantly expressed in yeast, SccAT and ScmAT, were also purified using this protocol.

The major band on SDS–PAGE gels of crude lysates, as well as after nickel-affinity purification of TbmAT, was *ca.* 55 kDa. This is consistent with the molecular weight of the GroEL chaperone that was co-expressed. The band was reduced to <5% of total protein after successive incubations of the column in buffer containing little or no imidazole. The optimized purification protocol was (1) overnight incubation after the first wash with buffer containing 20 mM imidazole, (2) a wash with 35 mM imidazole, (3) a wash with no imidazole followed by two 1 h incubations and a third overnight incubation, and (4) a final wash with no imidazole followed by elution of the pure protein with 500 mM imidazole.

All purification steps for untagged PaAT (expressed from pKMK4 or pKME1) were done on an AKTA FPLC (GE Healthcare). The clarified lysate was dialyzed against buffer containing 5 mM potassium phosphate, pH 6.8, 10 μM PLP and 1 mM DTT for 2 h. Dialyzed lysate was loaded onto a Macro-Prep ceramic hydroxyapatite column and eluted with a gradient to 200 mM potassium phosphate, pH 6.8. Fractions containing PaAT were identified by MDH-coupled activity assays [26]. PaAT-containing fractions were loaded onto a pre-packed MonoQ column equilibrated with 50 mM tricine, pH 8.0 with 10 μM PLP. PaAT does not bind to the MonoQ column under these conditions, but many contaminants do. The flow-through from this step was concentrated by ultrafiltration with Millipore Amicon 10,000 MWCO filters, and diluted into 50 mM tricine, pH 9.0 with 10 μM PLP to give a final pH of 8.8–9.0. The protein was loaded onto the MonoQ column equilibrated to pH 9.0 and eluted with a pH gradient down to 8.0. A final purification step on a pre-packed Sephadex200 column was necessary if the protein had been expressed from pKMK4.

Kinetic assays and data fitting

AATase activity was measured by the MDH-coupled assay described above except that the concentrations of L-aspartate and αKG were varied in order to obtain a range

of both substrate concentrations. All measurements were made on an Agilent 8453 UV-Vis spectrophotometer or SpectraMax 190 UV-Vis plate-reader (Molecular Devices) at 25 °C.

Kinetic data were fit with the SAS (SAS Institute, Cary, NC) or Origin (OriginLab, Northampton, MA) applications to equation 1 for a ping-pong bi–bi reaction [28]:

$$v = \frac{k_{cat}[E][Asp][\alpha KG]}{K_m^{Asp}[\alpha KG] + K_m^{\alpha KG}[Asp] + [Asp][\alpha KG]} \quad (1)$$

Results

The likelihood of success of any single attempt of heterologous expression of a given protein is unpredictable, because of the myriad differences between the originating organism and the transformed host. As the genes that were cloned in this study are from nine different organisms, including representatives from bacteria, protozoa, fungi, plants and animals, many challenges were encountered. Some of the solutions developed in response to difficulties during the cloning stage are explained below. Expression and purification of the proteins proved to be especially difficult for the majority of cases, especially when an intein-tag was present. Some of these difficulties are described below. Nonetheless, eleven out of the twelve targeted enzymes were ultimately purified to >85% purity (Fig. 3).

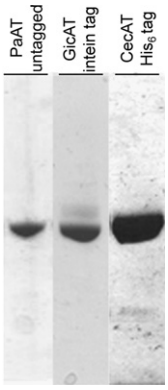

Fig. 3. Purity of enzyme preparations. A composite of three representative SDS–PAGE gels demonstrating sample purity of enzymes with different affinity tags is shown. (Left lane) Untagged PaAT expressed from the pKK223-3 construct; (center lane) GlcAT expressed from the pTYB construct after cleavage of the intein tag; (right lane) His_6-tagged CecAT expressed from the pET-43.1a construct. Load and migration are not equivalent between lanes.

Cloning aminotransferase genes

PCR amplification was straightforward for seven of the eight genes cloned from plasmid sources. The exception was *A. thaliana* cytosolic aminotransferase (AtcAT), for which PCR yielded an impure product. Thus, further amplification of the desired gel-purified fragment was required to improve the yield and purity of the correct product. PCR amplification of three aminotransferases from genomic DNA required much more troubleshooting. The *V. cholerae* and *C. trachomatis* genes (VcAT and CtAT) could only be amplified with Taq polymerase and in the presence of high concentrations of magnesium ion. Amplification of *P. aeruginosa* aminotransferase (PaAT) required 10% glycerol.

The combination of low-fidelity Taq polymerase and high [Mg^{2+}] predictably resulted in point mutations throughout the CtAT and VcAT genes. No sequences could be found that were identical to the published gene sequences for either CtAT or VcAT. Yet, certain base changes that were different from the expected sequence were repeatedly seen in multiple clones, implying that these base changes reflected the true wild-type sequence. It was possible to identify the wild-type sequence for each gene by sequencing multiple clones.

Five silent codon changes and seven coding base changes were discovered that differentiate the CtAT gene in strain L2[4] from the ortholog that was in the sequence databases at the time (from strain D/UW-3/CX). Since then, the L2 genome has been sequenced by the Sanger Institute and confirms these differences.

The published VcAT gene sequence [29] includes 11 additional codons at the 5′ end of the gene that we were unable to find in any clones. Because the published gene sequence was used for PCR primer design, the mutagenic PCR conditions were complicated (and most likely caused) by a completely non-specific 5′ primer. Dozens of clones had to be analyzed by DNA digestion to find those consistent with the correct gene before they were sequenced. The genome sequence has since been updated by The Institute for Genomic Research to reflect this altered 5′ end (TIGR Locus VC_A0513).

Enzyme preparations

Multiple expression systems were evaluated in order to obtain sufficient quantities of pure aminotransferases. PaAT was the only one for which purification of native, untagged protein was attempted. Although it was not feasible to develop individual purification protocols for the many aminotransferases studied, the purification of native and affinity-tagged PaAT allowed us to examine the effect of an affinity tag on aminotransferase activity. Heterologous expression of C-terminal tagged aminotransferases in *E. coli* was done with either the IMPACT-CN system (intein-tag) or pET-based histidine-tagged constructs (His_6-tag). The His_6-tag remains intact, while the intein-tag is cleaved during purification. Similar to the comparisons made with PaAT, kinetic characterization was carried out on two enzymes from both expression vehicles in order to evaluate the effects of the C-terminal tag. The *S. cerevisiae* aminotransferases (SccAT and ScmAT) were successfully expressed in and purified from yeast. AtcAT

expression was unsuccessful in *E. coli* or *S. cerevisiae*. Consequently, pure enzyme was obtained for eleven of the twelve aminotransferases, with AtcAT being the exception.

Native enzyme

Expression of native, untagged PaAT was tested for several plasmids varying in their origin of replication and promoters (see Materials and methods). Protein yields were about 1 and 30 mg/L from the pKK223-3 (P_{tac}) and pET-19b (P_{T7lac}) constructs, respectively. Purification from the latter expression system was possible in three steps instead of the four required for that expressed from pKK223-3.

IMPACT-CN system (intein-tag)

The IMPACT-CN vectors, pTYB1 and pTYB1-4 (Fig. 1), fuse an intein and a chitin-binding domain (CBD) to the target protein. The cytosolic aminotransferases from *Giardia intestinalis* and *T. brucei* (GicAT and TbcAT, respectively) expressed well from the intein construct. Mitochondrial *A. thaliana* enzyme (AtmAT), CtAT, and *P. falciparum* cytosolic aminotransferase (PfcAT) could be expressed at low levels with the intein-tag. However, highly purified protein could not be obtained for these three.

His_6-tagged enzyme from E. coli

The enzymes that expressed from the intein constructs (AtmAT, CtAT, GicAT, TbcAT, and PfcAT), as well as three others that did not express with the intein (from *C. elegans* cytosol (CecAT), *T. brucei* mitochondria (TbmAT), and VcAT), were expressed and purified via the pET-based constructs. Only His_6-tagged TbmAT required coexpression with chaperones to obtain high levels of enzyme. Production of a small amount of soluble ScmAT and AtcAT in *E. coli* was documented by anti-His_6 western blot, but the quantities were insufficient for verification by SDS–PAGE or AATase activity (data not shown).

Comparison of preparations from E. coli

PaAT was purified in its native form (from both the pKK223-3 and pET-19b constructs) as well as with a C-terminal His_6-tag. Yields of His_6-tag enzyme were comparable to those for native expression from pET-19b; 11 mg of the former was obtained per liter culture after 5 h expression at 37 °C while native enzyme was expressed at 30 °C for 6 h, and a quantity of 30 mg was purified per liter (see Materials and methods and Table 3). Similarly, GicAT and TbcAT were purified after expression from two different constructs. The lower yields of His_6-tagged compared to intein-tagged enzyme may be attributed to shorter expression times and, for TbcAT, a higher expression temperature. As in the case of these three His_6-tagged enzymes, streamlined expression methods were often adopted at the possible expense of protein yield, especially following observation of high expression levels for the native and intein-tagged enzymes (see Materials and methods and Table 3).

Table 3
Summary of aminotransferase expression

Enzyme	Successful expression vectors	Affinity tag (C-terminal)	Yield[a] (mg protein/L culture)
AtmAT	pET43.1a	His_6	1
CecAT	pET43.1a	His_6	3
CtAT	pET43.1a	His_6	11
GicAT	pTYB1	Intein-CBD	10
	pET43.1a	His_6	7
PaAT	pKK223-3	none	1
	pET-19b	none	30
	pET-19b	His_6	11
PfcAT	pET43.1a	His_6	5
ScmAT	pVV214	His_8	0.6
SccAT	BG1805	His_6-HA-ProteinA	3
TbcAT	pTYB1	Intein-CBD	30
	pET43.1a	His_6	4
TbmAT	pET43.1a	His_6	30
VcAT	pET43.1a	His_6	30
AtcAT	None		

[a] See Materials and methods for details of the expression and purification that may influence yields.

The C-terminal His_6-tags do not significantly affect substrate specificity ratios (data not shown), and generally result in enzymes with higher k_{cat} values (Table 4). The effect on k_{cat} probably reflects the shorter time needed for purification of His_6-tagged proteins compared to the intein or native methods.

Expression and purification of enzyme from S. cerevisiae

Both of the *S. cerevisiae* class Iα aminotransferases were recombinantly expressed in yeast. The putative mitochondrial signal sequence (see Materials and methods) was removed from the ScmAT gene during cloning to enable cytosolic expression of the enzyme, and an eight histidine

Table 4
Comparison of His_6-tagged aminotransferase activities with those of their native counterparts[a]

Aminotransferase	C-terminal Tag	K_m^{Asp} (mM)	k_{cat} (s^{-1})	k_{cat}/K_m^{Asp} ($M^{-1}\ s^{-1} \times 10^{-3}$)
GicAT	Intein (cleaved)	18	71	4.0
		(2)	(3)	(0.4)
	His_6	9.0	93	10.2
		(0.5)	(3)	(0.6)
PaAT	None (pKK223-3)	1.5	58	40
		(0.5)	(8)	(10)
	None (pET-19b)	1.8	60	40
		(0.5)	(10)	(10)
	His_6	2.1	99	47
		(0.2)	(4)	(5)
TbcAT	Intein (cleaved)	8.5	29.8	3.5
		(0.6)	(0.5)	(0.1)
	His_6	9.6	104	11
		(0.8)	(4)	(1)

[a] Standard errors are in parentheses.

tag was fused to the C-terminus. The SccAT construct contained multiple tandem C-terminal affinity tags, including six histidines. Both enzymes were purified to homogeneity by nickel affinity chromatography.

Spectrophotometric characterization

All of the investigated aminotransferases are colorless at pH 8.0 with the exception of VcAT, which is yellow. The maximum absorption of VcAT of 432 nm is indicative of the protonated PLP internal aldimine. The other enzymes have maximum absorbances between 350 and 365 nm at pH 8.0, the signature of unprotonated PLP internal aldimine [30].

Discussion

The objective of this study was to increase the diversity of aminotransferases for which kinetic data are available. The amino acid sequences of kinetically characterized aminotransferases were compared to those in a sequence database to identify divergent proteins. The details of this selection method, as well as a detailed analysis of the protein sequences, will be presented elsewhere (K.E. Muratore, J.R. Srouji, B.E. Engelhardt, M.I. Jordan, S.E. Brenner and J.F. Kirsch, manuscript in preparation).

A consequence of the selection method is that the genes investigated here are endogenous to a broad range of species, and, thus, codon usage and other species-specific expression parameters vary. Therefore, finding appropriate expression vehicles presented significant individual challenges. As the N-termini of aminotransferases are involved in homodimer formation [31,32], it is preferable to attach affinity tags to the C-termini.

Heterologous expression

Histidine and intein C-terminal tags were employed to aid in purification. A positive feature of the IMPACT-CN (intein) expression system is that the affinity tag can be cleaved cleanly from either terminus, without leaving residual non-native residues [33]. However, the potential benefits of the latter vehicle were largely unrealized in this study. Two of the ten intein-tagged aminotransferases (GicAT and TbcAT) were easily expressed and purified. Two others expressed poorly and showed evidence of apparent cleavage of the tag *in vivo*. These two problems obviated purification to useful levels—purity was <5% for both intein-tagged CtAT and AtmAT. Additionally, cleavage of the intein from CtAT was inefficient and time-consuming. PfcAT expressed poorly, and the tag could not be cleaved. The remaining five intein-tagged aminotransferases could not be expressed.

The observed expression problems are not due entirely to the aminotransferase genes, since all but two expressed well from the pET-43.1a (His_6-tagged) construct. The intein tag is larger than an aminotransferase monomer (59 kDa *vs.* ~45 kDa), and is not optimized for *E. coli* expression. Intein-tagged PfcAT could only be expressed in the presence of rare tRNAs or chaperones, but expression of His_6-tagged PfcAT required neither one. These results suggest that unoptimized target genes used in combination with native intein tags may have a larger negative effect on expression than would be anticipated from the results of the separate expression of each gene.

Due to the number of enzymes studied here, an *E. coli* strain that allowed rapid detection of expressed enzymes was desirable. Strain MG204, which has low endogenous AATase activity in crude lysates, is excellent for screening, but is not optimized to express proteins with specialized tags such as the intein. It is likely that optimization of the codon usage and/or *in vivo* solubility of the intein for *E. coli* [34] would reduce the encountered problems in expression.

Affinity purification

Some of the intein cleavage problems were addressed by the manufacturer [33]. They stated that the residue immediately preceding the C-terminal tag affects both premature *in vivo* cleavage as well as *in vitro* cleavage efficiency. This was evaluated by varying this amino acid in maltose-binding protein that was fused to the intein. This residue is isoleucine in PfcAT, and is serine in both AtmAT and CtAT. Isoleucine is expected to cleave *in vitro*, but the intein could not be cut from PfcAT. Serine should have better *in vitro* cleavage efficiency (50–74%) and less *in vivo* cleavage (<10%) than what was observed for AtmAT and CtAT.

The inconsistency between these results and the manufacturer's evaluations of maltose binding protein expression and purification implies that cleavage efficiency is dependent on additional factors possibly including local structure or the extended sequence upstream of the cleavage site. This hypothesis gains support from an experiment in which the tripeptide Gly-Arg-Ala was inserted between the intein and a *Neisseria meningitidis* protein for improved on-column cleavage [35]. Recently, Cui and co-workers [36] introduced additional cysteine residues in intein domains and observed reduced *in vivo* cleavage. Although both studies were done in pTWIN vectors, whose inteins differ from that of pTYB1, they support the hypothesis that cleavage efficiency is not dependent solely on a single adjacent residue.

Comparisons of the effects of the two tagging methods on enzyme activity were made for GicAT and TbcAT, both of which expressed well in both systems. The K_m values for the cleaved intein-tagged and His_6-tagged constructs are comparable, but the k_{cat} values are greater for His_6-tagged protein (Table 4). Similarly, the k_{cat} value recorded for His_6-tagged PaAT is about 2-fold greater than that found for native PaAT expressed from either the pET-19b or pKK223-3 constructs. Histidine-tagged proteins are normally purified within hours after lysis, while the protocols

for IMPACT-CN and native aminotransferase purifications generally take one day and three days, respectively.

Concluding remarks

Eleven aminotransferase genes were cloned for heterologous expression in *E. coli*, nine of which were successfully expressed and purified to homogeneity. One of the two that was not purified from *E. coli*, and a twelfth gene, were expressed and purified from *S. cerevisiae*—both are native to yeast. Histidine-tags on the C-terminus were beneficial because (1) they did not reduce expression of the desired product, as did large intein tags, and (2) the simple and fast purification protocol saved time and, apparently, enzyme activity.

To the best of our knowledge, this is the first attempt to prepare such a large number of homologs of the same enzyme from different species. The complete characterization of these aminotransferases, especially their substrate specificity, will greatly increase what is known about this ubiquitous protein family and aid in our understanding of enzyme evolution. The full elucidation of the substrate specificities for these enzymes will be reported elsewhere (K.E. Muratore, J.R. Srouji, B.E. Engelhardt, M.I. Jordan, S.E. Brenner and J.F. Kirsch, manuscript in preparation).

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