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Probing the active site of MIO-dependent 2,3-aminomutases, key catalysts in the biosynthesis of beta-amino acids incorporated in secondary metabolites

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Abstract

The tyrosine aminomutase *SgTAM* produces (*S*)- β -tyrosine from *L*-tyrosine in the biosynthesis of the enediyne antitumor antibiotic C-1027. This conversion is promoted by the methylideneimidazole-5-one (MIO) prosthetic group. MIO was first identified in the homologous family of ammonia lyases, which deaminate aromatic amino acids to form α,β -unsaturated carboxylates. Studies of substrate specificity have been described for lyases but there have been no reports in altering the substrate specificity of aminomutases. Furthermore, it remains unclear as to what structural properties are responsible for catalyzing the presumed readdition of the amino group into the α,β -unsaturated intermediates to form β -amino acids. Attempts to elucidate specificity and mechanistic determinants of *SgTAM* have also proved to be difficult as it is recalcitrant to perturbations to the active site via mutagenesis. An X-ray co-crystal structure of the *SgTAM* mutant of the catalytic base with *L*-tyrosine verified important substrate binding residues as well as the enzymatic base. Further mutagenesis revealed that removal of these crucial interactions renders the enzyme inactive. Proposed structural determinants for mutase activity probed via mutagenesis, time-point assays and X-ray crystallography revealed a complicated role for these residues in maintaining key quaternary structure properties that aid in catalysis.

INTRODUCTION

MIO-dependent aminomutases are responsible for the conversion of α -amino acids to β -amino acids, which are incorporated in a variety of natural products.¹ This non-trivial transformation occurs via a 2,3-amine shift with the assistance of the MIO prosthetic group (4-methylideneimidazole-5-one, Figure 1A), an electrophilic moiety formed by the self-condensation of an Ala-Ser-Gly motif in the active site of the enzymes.^{2, 3} The catalytic mechanism of the MIO-containing enzymes has been an active field of study, with a majority of mechanistic and structural studies focused on MIO-dependent ammonia lyases.⁴ MIO-dependent lyases catalyze the deamination of *L*-histidine, *L*-phenylalanine, and *L*-tyrosine generating the corresponding α,β -unsaturated acid (Figure 1B). More recently, novel chemistry has been attributed to MIO-containing enzymes, first with the characterization of *SgTAM*, which is responsible for the biosynthesis of (*S*)- β -tyrosine in the secondary metabolic pathway to the antitumor antibiotic C-1027 in *S. globisporus* (Figure 1C).^{5, 6} Since this initial discovery, additional *L*-tyrosine and *L*-phenylalanine aminomutases have been described.^{7–11} The chemical mechanism by which MIO-containing enzymes catalyze the conversion of *L*-amino acids to their lyase or mutase products is debated in the literature.^{1, 4} Two general mechanisms have been proposed; the first proceeds

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through an amino-MIO adduct mechanism via electrophilic addition of the substrate α -amino group into the prosthetic group. Deprotonation at the β -position and elimination occurs on the substrate/enzyme adduct through an E1-cb type mechanism.¹² A second proposed mechanism involves electrophilic activation (Friedel-Craft type) of the aromatic side chain of the α -amino acid substrate, leading to removal of the β -proton through a carbocation intermediate, liberating ammonia.³ In either scenario, aminomutase chemistry could result from re-addition of the tethered (for the E1-cb mechanism) or free ammonia (Friedel-Craft type) into the lyase product (Figure 1C).

X-ray crystal structures of several MIO-lyases have been determined, and all share a common overall structure that is primarily α -helical and a homotetramer.^{2, 13–17} The active site, containing the MIO prosthetic unit, is located at the interface of three of the four monomers and at the positive termini of multiple α -helices. The structure of the *SgTAM* demonstrated that the overall and active site architecture of aminomutases is very similar to MIO-dependent lyases.¹⁷ These similarities suggest that the two classes act through a similar mechanistic pathway. Indeed, *in vitro* experiments show that *SgTAM* produces the lyase product 4-hydroxycinnamic acid along with the biologically relevant product β -tyrosine and, over time, the lyase product predominates in the aminomutase reaction mixture.⁵

The structural characterization of *SgTAM* provided significant insights into the mechanism of MIO-dependent aminomutases.^{17, 18} For example, Tyr63 was identified as critical in the catalytic pathway by acting as the enzymatic base.¹⁹ Additionally, residues important for substrate discrimination were proposed (Figure 2). The X-ray structure revealed limited differences around the active site between this *L*-tyrosine aminomutase and the *L*-tyrosine lyase *RsTAL*. The only clear variation in residues in close proximity to the MIO group is at position 415 (numbering from *SgTAM*) where *SgTAM* had a Tyr at this position and *RsTAL* a Val. Additional differences noted between the two structures were in a tunnel region leading to the active site from bulk solvent. In the aminomutase, residues Glu71 and Tyr303 appear to close the tunnel to the active site, a feature that is not present in the more open tunnel in the *RsTAL* structure. The residues that block the opening to the active site are believed to sequester ammonia and/or *L*-tyrosine in the active site so that the complete transformation to β -tyrosine can occur, instead of immediately releasing 4-hydroxycinnamic acid and ammonia. The structural determinants for lyase and aminomutase chemistry remain to be determined. A recent report examined the effects of mutations on CdmF, an *L*-tyrosine aminomutase responsible for the production of (*R*)- β -tyrosine in the chondramide family of natural products.¹⁰ Conserved sequence motifs from lyases were exchanged into CdmF in an effort to shift function to ammonia lyase chemistry. These experiments resulted largely in either no enzyme activity or substantially reduced activity with retained aminomutase activity. One notable mutant exchanged into CdmF a homologous region from the (*S*)-tyrosine aminomutase MdpC4. Unpredicted, this alteration, which involved residues not directly involved in catalysis or substrate binding, shifted the chemistry from aminomutase to ammonia lyase activity.

This manuscript describes biochemical and structural investigations into the mechanism of *SgTAM*. Residues around the active site predicted to be important in catalysis and substrate recognition were probed with site-direct mutagenesis followed by biochemical and structural characterization of the mutants. The results provide insight into the overall chemistry and identify several residues that are critical to overall function.

RESULTS AND DISCUSSION

The role of Tyr63 as a catalytically-relevant enzyme base

The co-complex structure of *Sg*TAM bound to a product analog (α,α -difluoro- β -(*S*)-tyrosine) placed Tyr63 adjacent to the bound analog.¹⁹ In particular, the phenol of Tyr63 was observed positioned in close proximity to the substrate's α - and β -hydrogens. The Tyr63 residue is located on a loop covering the active site that is frequently disordered and therefore not observed in homologous MIO-dependant lyase crystal structures. The aminomutase reaction involves exchange of the α - and β -hydrogens and labeling evidence suggests the exchange is conserved.^{20, 21} Based on this orientation, Tyr63 was predicted to be the enzymatic base in *Sg*TAM responsible for facilitating the deprotonation/reprotonation.¹⁹ The relatively high pKa's of the substrate hydrogens suggests that an uncommon ionization state of Tyr63 may be present, and indeed a phenolate form of tyrosine would be stabilized by the positive ends of six α -helices¹³ and a conserved hydrogen-bond to the backbone N-H of Gly70. Previous biochemical characterization of a Tyr63Phe mutant showed no turnover of the substrate.¹⁹ Likewise, mutation of the homologous Tyr in *Rs*TAL (Tyr60) to Phe resulted in a complete loss of activity.²²

To further probe the role that Tyr63 plays in the function of MIO-based enzymes, we solved the X-ray crystal structure of Tyr63Phe *Sg*TAM in the presence of substrate *L*-tyrosine. The mutant was purified and crystallized in the presence of 2.0 mM free *L*-tyrosine. Crystals grew under similar conditions as the crystals of wild-type enzyme and diffracted to a resolution of 2.3 Å. The structure of the co-complex provides additional evidence regarding the importance of Tyr63 and insights into substrate binding. Overall, the structure is very similar to the unliganded structure (bound to β ME) with an overall RSMD (all atoms) of 0.39 Å. The area around the MIO prosthetic group is very similar to previous structures and the mutation at position 63 is clearly evident in the maps (Figure 3A,B). This congruence suggests that the mutation does not affect the overall structure of the enzyme. A partially occupied free *L*-tyrosine can be modeled into the density maps bound to the MIO in a manner similar to that observed for the product analog, α,α -difluoro- β -(*S*)-tyrosine (Figure 3C).¹⁹ The partial occupancy of the substrate can be attributed to the low solubility of *L*-tyrosine in our crystallization mixture and the propensity of the MIO prosthetic group to be inactivated by nucleophiles. In addition, it is possible that not all of the four active sites are active catalysts. Although density for the α -carbon of *L*-tyrosine is weak, the MIO is clearly bound to a ligand in both the Fo–Fc and 2Fo–Fc maps and the maps are best modeled as an overlay of *L*-tyrosine and noncovalently bound waters. The model confirms the importance of His93 and Tyr415 in interacting with the side chain of the substrate and Arg311 in forming a salt bridge with the α -carboxylate. Relevant for the catalytic mechanism, the similarity of the substrate-like and product-like structures support a catalytic mechanism where the base (Tyr63) is positioned in between the α - and β -hydrogens assisting in a low-barrier transfer of hydrogen atom through an anionic intermediate.¹ Furthermore, the positioning of the substrate tethered to the MIO via the amine lends support to the E1-cb mechanism.

Amino acid substrate specificity of *Sg*TAM

The substrate specificity of MIO-dependent enzymes is limited to the aromatic amino acids: *L*-tyrosine, *L*-histidine and *L*-phenylalanine. X-ray structures of representative examples have established that the substrates bind in similar orientations with the side chain interacting with conserved regions of the active site.¹ Based on co-crystal structures of *L*-tyrosine specific *Sg*TAM and *Rs*TAL with substrate-like inhibitors, a histidine residue (His93 and His89, respectively) is within hydrogen bonding distance to the phenol of the substrate *L*-tyrosine.^{14, 19} In contrast, the *L*-phenylalanine specific MIO-lyases lack a hydrogen-bond

donor/acceptor at this position and in place have a conserved Phe.^{14, 23} The importance of this interaction on substrate discrimination has previously been probed in *RsTAL*, where the His89Phe mutant changed specificity from L-tyrosine to L-phenylalanine. Based on significant structural homology to *RsTAL*, we anticipated that the corresponding point mutation would similarly affect substrate selectivity in *SgTAM*. The His93Phe *SgTAM* mutant was constructed and assigned for activity with L-phenylalanine and L-tyrosine as potential substrates. Surprisingly, the His93Phe *SgTAM* mutation had no activity for either substrate under all conditions tested. The lack of activity could be attributed to improper folding of the mutant, however comparison to the wild-type enzyme with circular dichroism spectrometry was consistent with a well-folded protein (data not shown). In general, efforts to measure the low turnover of altered substrates are likely hampered by slow catalytic efficiency of *SgTAM*. Mutase activity of *SgTAM* is 160–300 times less efficient than *RsTAL* lyase activity and perturbations to the active site could easily extinguish activity overall.^{14, 23} Additionally, it was predicted based on spectroscopy that *SgTAM* only has about 18% of possible MIO formed.⁵ Minor changes in the active site might perturb the structure enough to eliminate nearly all MIO formation.

The structural basis of aminomutase / lyase chemistry in *SgTAM*

The X-ray structure of the L-tyrosine-specific MIO-lyase *RsTAL* provides a model to probe the structural basis for aminomutase function in *SgTAM*. Based on the general rarity of aminomutases versus lyases, it is reasonable to predict that the former evolved from the later. The question of the molecular and structural basis for the added chemistry of aminomutases resulting in the 2,3-amino shift is intriguing. As mentioned, there are minimal differences between the active sites of the aminomutase *SgTAM* and the lyase *RsTAL* and the primary difference being position 415 in *SgTAM* (Figure 2). The aminomutase has a Tyr and the lyase has a Val. The other significant structural difference in between *SgTAM* and lyases is a more closed and restricted active site, in particular a tunnel leading to the MIO prosthetic group. We first probed the effect of position 415 on the activity of *SgTAM*; it was anticipated that Tyr415Val *SgTAM* would exhibit lyase activity (producing solely 4-hydroxycinnamate) as opposed to aminomutase activity. Unfortunately, this mutation resulted in an enzyme lacking any activity even after 24 hours incubation time. We postulated that the mutation resulted in a change in active site hydrophobicity that precluded binding of L-tyrosine, but not enough to accommodate L-phenylalanine binding. Therefore, the double mutant Tyr415Phe/His93Phe was constructed in order to push the activity of *SgTAM* to a L-phenylalanine lyase. Again, no activity toward L-phenylalanine was observed. In the case of these mutant constructs, a change in protein folding could be playing a role as attempts at crystallizing the mutants failed where other point mutants easily crystallized under the same conditions as the wild-type.

As direct changes to the active site of *SgTAM* had a negative effect on the chemistry, we turned to addressing the solvent accessibility of the active site. *RsTAL*, as discussed above, has a more open active site when compared to *SgTAM* and the MIO prosthetic group has increased exposure to outside solvent.¹⁴ It was proposed that an open active site decreases the ability of the enzyme to maintain 4-hydroxycinnamate and ammonia in the active site leading to release of the lyase products.¹⁹ The closed active site of aminomutases like *SgTAM* could retain ammonia in the active site pushing the chemistry to readdition and formation of (S)- β -tyrosine.

The X-ray structure of *SgTAM* showed that two residues (Glu71 and Tyr303) form a hydrogen bond and appear to contribute to blocking solvent access to the active site.¹⁷ In order to probe the role of accessibility of the active site as the aminomutase/lyase determinant, we made rational mutations in this region. These tunnel mutant constructs involved not only decreasing the steric bulk in the region, but also removing the hydrogen

bond and included Glu71Ala, Tyr303Ala, and the double mutant. Despite a general decrease in activity, all three mutants showed lyase activity over time and still produced some amount of β -tyrosine (Figure 4ABC). The Glu71Ala mutant had the biggest decrease in activity of the two single mutants; the percent of HCA and β -tyrosine both never surpassed 15% even after 24 hrs, with β -tyrosine forming in excess of HCA the entire time. The effect of the Tyr303Ala mutation was minor as a similar time curve was observed to the wild-type enzyme, though wild-type converted as much as 60% of tyrosine to β -tyrosine, while the mutant peaked just under 40%. The double mutant resulted in roughly the same time course as the single Glu71Ala mutant.

To further investigate the low activity of the Glu71Ala mutant, we solved the structure to a resolution of 2.3 Å. Figure 4D shows a cutaway view of the wild-type *SgTAM* active site, including the closed tunnel. When Glu71 is mutated to an alanine, the tunnel is open and the active site is solvent exposed (Figure 4E). It was expected that opening the tunnel, like the tunnel from *RsTAL* (Figure 4F), would speed up the release of ammonia or 4-hydroxycinnamate before the ammonia could add back into cinnamate to form β -tyrosine. This activity was not observed for the Glu71Ala mutant nor for the double mutant. It appears that *SgTAM* still acts as a mutase, but its overall rate of turnover is decreased, perhaps a result of improper formation of the active unit. The biological unit is composed of four monomers, three of which contribute residues to the active site; residues 71 and 303 within one tunnel are from two different monomers. Perhaps problems with the interfacing of the monomers are to blame for the lowered activity.

CONCLUSION

The chemistry of the MIO prosthetic group is unique and several unanswered questions remain concerning the mechanism. Ammonia lyases catalyze the elimination of ammonia from aromatic amino acids. The challenge a lyase catalyst must overcome is the significantly low acidity of the β -hydrogens. There is not a general consensus in the literature on how the electrophilic MIO group accomplishes this elimination. The discovery that MIO-dependent enzymes can also promote an overall aminomutase reaction added a further twist on the mechanism of this enzyme family. Previously characterized aminomutases use multiple metals/cofactors to catalyze the 1,2-amino shift via radical intermediates. Here we reported the structural and biochemical analysis of rationally designed mutants of the *L*-tyrosine aminomutase *SgTAM*. The constructs were designed to probe the mechanism of substrate recognition and the basis for aminomutase chemistry. One residue proposed to be directly involved in the chemistry is Tyr63. Previous X-ray crystal structures of *SgTAM* bound to product analogs placed this residue approximately equidistant from the α - and β -hydrogens of *L*-tyrosine. The complete inactivity of the Tyr63Phe mutant provides an opportunity to obtain a co-crystal structure of *SgTAM* bound to the substrate. Indeed the co-crystal structure of *SgTAM* / *L*-tyrosine confirmed the proposed role of Tyr63 in shuttling the proton between the α - and β -positions. The substrate-bound structure complements our previous structures of product-analog providing a comprehensive picture of active site residues involved in substrate recognition. In addition to the structural basis of substrate recognition and chemistry, we were interested in probing the nature of the aminomutase activity in *SgTAM*. A majority of known MIO-dependent enzymes are ammonia lyases and evidence suggests that lyases and aminomutases share a similar catalytic pathway. As the differences in structures of lyases and aminomutase with the same substrate are minimal, the possibility to switch the chemistry is alluring and would provide significant insights into enzyme function. We therefore attempted to shift the activity of *SgTAM* toward that of an ammonia lyase, presumably through the loss of mutase activity (see Figure 1B). Although several changes were made based on clear structural differences between *SgTAM* and *RsTAL*, the desired switch from aminomutase to lyase was

not observed. Indeed, a majority of mutants (several not presented, including mutants of Y308)) resulted in decreased or complete loss of activity. Of note, alterations to the accessibility of the active site to solvent did not have the predicted effect on function and slowed down activity overall, perhaps by hindering complete assembly of the catalytically relevant tetramer or the MIO prosthetic group. Mutant versions of *SgTAM* structurally similar to *RsTAL* were verified with X-ray crystallography, but retained an aminomutase reaction profile albeit with reduced function. Overall, the negative results are likely a result of the highly reactive nature of the MIO prosthetic group. Alterations around the active site region can affect the self-catalyzed condensation that forms the electrophile and also compromise the protection of the MIO from extraneous nucleophiles. Further investigations are needed to establish the detailed catalytic mechanism and the nature of the lyase and aminomutase chemistry.

MATERIALS AND METHODS

General

PfuUltra, DpnI and other reagents for site-directed mutagenesis were ordered from Stratagene (La Jolla, CA). Oligonucleotide primers were ordered from IDT DNA (Coralville, IA). T4 DNA ligase and restriction enzymes were purchased from New England Biolabs (Ipswich, MA). DNA sequencing was done by Genewiz (S. Plainfield, NJ). Thrombin, IPTG, LB media, antibiotics and other reagents were from Sigma-Aldrich (St. Louis, MO) or VWR (West Chester, PA).

DNA manipulation

Mutant constructs of *SgTAM* from *Streptomyces globisporus* (Locus AY048670) were produced from the plasmid expression construct pBS1022 (in pET-28a in *NdeI-HindIII* sites) using a modified version of the Quikchange site-directed mutagenesis protocol as described previously.⁵ Mutants were produced using their corresponding primer pairs as follows:

Mutation	Forward Primer	Reverse Primer
Y63F	5'-CAGAACATCCCCATCTTCGGGGTGACCAACCGGG-3'	5'-CCCGGTGGTGACCCCGAAGATGGGGATGTTCTG-3'
E71A	5'-CCGGGTACGGCGCGATGATCTACATGC-3'	5'-GCATGTAGATCATCGCGCCGTACCCGG-3'
E71A,Y303A	5'-CCGGGTACGGCGCTATGATCTACATGC-3'	5'-GCATGTAGATCATAGCGCCGTACCCGG-3'
H93F	5'-CCAATCTCGTCCGTAGCTTCAGCGCGGGAGTCGGTCC-3'	5'-GGACCGACTCCCGCGCTGAAGCTACGGACGAGATTGG-3'
Y303A	5'-GCGCTCGGAGATCGCCCTGCAGAAGGCC-3'	5'-GGCCTTCTGCAGGGCGATCTCCGAGCGC-3'
Y415V	5'-CGCCGGCGCCAGGTCGCCGCCACCGC-3'	5'-GCGGTGGCCGGGACCTGGGCGCCGCGC-3'

The Tyr303Ala mutant was produced using the procedure from the Quikchange handbook. Modifications to the published protocol for the remaining mutants were as follows: the Tyr63Phe mutant was produced using 1X reaction buffer, 100 ng plasmid, 500 ng forward and reverse primer, 200 μ M dNTPs, 5% DMSO, and 2.5 U *PfuUltra*, with the following thermal cycler parameters: 95 °C for 5 min; 35X (95 °C for 30 sec, 62 °C for 45 sec, 72 °C for 10 min); 72 °C for 10 min; 4 °C. The remaining mutants had slightly altered procedures: Tyr415Val had an extension time of 8 min, Glu71Ala was cycled 20X to lower the chance of random annealing and had an extension time of 10 min. To produce the Glu71Ala/Tyr303Ala double mutant, new primers were used with the Tyr303Ala mutant (E71A, Y303A) because the original could not again successfully produce the mutation despite repeated attempts. The reaction was cycled 20X with an extension time of 10 min,

producing the double mutant. Following mutagenesis reactions, 2 μ L of DpnI was added to the reaction mixture and incubated for 2 h at 37 °C. The QIAquick PCR purification kit was used to purify plasmid DNA, which was eluted with 30 μ L H₂O. To close nicks resulting from mutagenesis, a ligation was performed overnight with 1 μ L T4 DNA ligase, 1X T4 DNA ligase reaction buffer at 16 °C. Typically, 1 μ L of the ligation mixture was transformed into NovaBlue component *E. coli* cells, then grown overnight in 5 mL LB/kanamycin broth. Plasmids were isolated using the QIAprep Spin MiniPrep Kit and presence of mutations were confirmed by sequencing. Subcloning was used to produce the Tyr93Phe/Tyr415Val mutant; the Tyr93Phe and Tyr415Val plasmids were digested with *Nde*I and *Sac*I. The DNA was purified via 1% agarose gel and isolated using the QIAquick Gel Extraction Kit and ligated with T4 DNA ligase as described.

Enzyme production and purification

Mutant *Sg*TAM constructs were transformed into BL21(DE3) *E. coli* cells. One colony was used to inoculate 5 mL of LB/kanamycin media and after overnight growth used to inoculate 1 L of LB/kanamycin media. Growth continued until O.D.=0.6 with shaking (37 °C at 150 RPM). Overexpression was induced by addition of 50 μ M IPTG and the culture was incubated overnight at 18 °C. Cells were collected by centrifugation (20 min, 2000 g), resuspended in 25 mL of lysis buffer containing 20 mM Tris-HCl, pH 7.5 and 500 mM NaCl, and frozen (−78 °C). The cell pellet was thawed and lysed by two passes through a French Press at 1000 PSI. The lysed cells were then centrifuged (20 min, 10,000 g) to remove cell debris and the protein was purified batch-wise using Ni-NTA resin (Qiagen). For use in activity and binding assays, the purified protein was dialyzed into a low salt buffer (50 mM CHES-NaOH pH 9.0, 50 mM KCl) and was either used immediately or stored at −78 °C in 20% glycerol to be used for assays within one week. Protein for crystallography was dialyzed into low salt buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl), concentrated to ~1 mL, and the His₆-tag was cleaved using 5 U of thrombin in cleavage buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM CaCl₂) for 2 days at 4 °C. The protein solution was then diluted to 5 mL with lysis buffer and purified on a HiTrap-Q ion exchange column (0–1000 mM NaCl gradient in 50 mM Tris-HCl pH 7.5) followed by a Superdex 200 gel filtration column (20 mM Tris-HCl pH 7.5, 100 mM NaCl). The purified protein was concentrated to ~10 mg/mL for crystallography (a total yield of 4 mg/L of cells).

Biochemical assay of *Sg*TAM mutant constructs

The activity of *Sg*TAM mutants was measured using an HPLC based assay as described.⁵ Reactions were performed at 25 °C with 100 μ M L-tyrosine, 50 mM CHES-NaOH, 50 mM KCl, pH 9.0 and 0.5 mg/mL enzyme in a total volume of 1.5 mL. All mutant constructs were monitored for activity with time points at 1 h and 18 h. For Glu71Ala and Tyr303Ala mutants, the time course assay was run by quenching 50 μ L aliquots of reaction mixture by addition to a tube containing 2 μ L 0.5 M HCl. Time points were taken at 10 min, 30 min, then every half hour for 8 h, and then every hour until 12 h. Additional time points were taken after 24 h. Each aliquot was centrifuged for 1 min at 14,000 g to remove enzyme. The supernatant was subjected to o-phthalaldehyde (OPA) derivatization by increasing the pH to 10 with 2 μ L 1M NaOH followed by mixing with 50 μ L of fresh, degassed OPA reagent (4 mg OPA, 4.25 mL 100 mM sodium borate buffer pH 10.7, 50 μ L ethanol and 11 μ L β -mercaptoethanol) for 30 sec, and then quenched with 1 μ L glacial acetic acid. Derivatized samples were subjected to HPLC analysis on a VYDAC C18 Protein and Peptide column, monitoring at 330nm (eluent A: 5 mM sodium acetate pH 5.7 in with 5% THF; eluent B: 5 mM sodium acetate pH 5.7, 45:45:10 methanol/acetonitrile/water; gradient: 0–38 min at 10–50% B, 38–40 min at 50%, 40–52 min at 50–10% B, 52–57 min at 10% B at a flow rate of 1 mL/min).

Mutant constructs Tyr93Phe, Tyr415Val, and the double mutant of the two was tested for activity with L-phenylalanine under similar assay and analysis conditions.

Crystallization and X-ray data collection

SgTAM mutant constructs Glu71Ala and Tyr63Phe (both 58 kDA, 539 amino acids) were crystallized as described previously with some minor modifications.¹⁷ The Glu71Ala mutant was crystallized in 100 mM trimethylamine N-oxide (TMAO), 4.6 M sodium formate, at 4 °C. The Tyr63Phe mutant crystallized in 95 mM TMAO, 4.6 M sodium formate with 2 mM L-tyrosine (incubated with protein for two days at 4 °C), at 20 °C. Solutions were sterile filtered prior to use. Crystals typically formed within two days and reached full size within a few weeks.

Data was collected at the National Synchrotron Light Source at Brookhaven National Laboratories on beamlines X12C and X25, respectively (Table 1).

The structures were solved using molecular replacement with unliganded *SgTAM* (PDB code 2OHY) as the search model using MOLREP.²⁴ Model building was performed with the program COOT.²⁵ Refinement cycles and generation of electron density maps were done using the CNS suite of programs.²⁶ The parameter and protein topology files were modified to incorporate the MIO moiety into the protein backbone structure. Cycles of rigid body refinement, simulated annealing guided by s-weighted composite omit maps were carried out until the *R*-values were no longer improved to any extent. The program PyMOL (Delano Scientific, San Carlos, CA) was used to generate graphic images.

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Figure 1. The chemistry of MIO-dependent enzymes. A. The structure of the prosthetic group MIO. B. Schematic of the reactions catalyzed by lyases and aminomutases starting with an L-amino acid. C. The aminomutase *SgTAM* in the biosynthetic pathway of the antitumor antibiotic enediyne C-1027.

Figure 2.
The active site of the aminomutase, SgTAM. The MIO group is shown in red and residues in close proximity to the MIO are illustrated in dark grey (PDB code 2OHY) and schematic representation.

Figure 3.

The X-ray structure of the Tyr63Phe mutant *SgTAM*. A. Active site mutant with L-tyrosine modeled bound to the MIO. B. Representation of the interactions of the substrate with the enzyme. C. Electron density maps of the active site. An 2Fo-Fc map (blue, 1.5σ) and Fo-Fc (green, 3.0 σ) show partial occupancy of L-tyrosine. Maps were generated without L-tyrosine modeled.

Figure 4.

Probing the active site tunnel to the MIO prosthetic group in *SgTAM*. ABC. Time courses of the reaction of mutant enzymes with L-tyrosine as a substrate. The plots monitor change in concentration of substrate (■), lyase product 4-hydroxycinnamate (HCA, ●) and aminomutase product, (S)-β-tyrosine (▲). Cutaway views showing the active site and tunnel region of MIO-enzymes (probe sphere radius of 1.4 Å). D. Wild-type *SgTAM* (PDB 2OHY) with the MIO prosthetic group shown in red. E. Similar orientations of the Glu71Ala *SgTAM* mutant (PDB 3KDY) and F. The lyase *RsTAL* (PDB 2O6Y).

Table 1

X-ray crystallographic data collection and refinement statistics

	Tyr63Phe	Glu71Ala
PDB code	3KDZ	3KDY
Space group	P2 ₁ 2 ₁ 2	P2 ₁ 2 ₁ 2
Unit cell parameters (Å)	a=92.7,b=145.9,c=74.8	a=92.6,b=145.9,c=74.9
<i>Data collection</i>		
Resolution (Å)	50.0–2.20 (2.28–2.20)	50–2.20 (2.28–2.20)
Wavelength (Å)	0.9793	1
No. of reflections (measured/unique)	751714/52517	226063/50047
% Completeness *	99.9 (100.0)	95.2 (87.1)
R _{sym} *	0.087 (0.653)	0.086 (0.440)
I/σ *	30.0 (4.3)	11.0 (1.8)
Redundancy *	14.3 (14.2)	4.6 (4.0)
<i>Refinement</i>		
No. of reflections (total/test)	50153/5053	47693/4814
R _{work}	0.204	0.238
R _{test}	0.249	0.265
No. amino acids (chains A/B)	529/529	529/529
No. atoms		
Protein (chains A/B)	4012/4012	4010/4010
Ligands (L-tyrosine)	26	-
Water Molecules	216	468
Mean B (protein/Tyr/water) (Å ²)	42.9/30.8/37.8	39.3/-/38.3
RMSD from ideal geometry		
RMSD bonds (Å)	0.008	0.005
RMSD angles (°)	1.4	0.8
Ramachandran plot by PROCHECK (%)		
Core region	89.8	87.0
Allowed region	10.2	12.7
Generously allowed	0.0	0.2
Disallowed	0.0	0.0

* overall (highest resolution shell)