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# Assessing the Deamination Rate of a Covalent Aminomutase Adduct by Burst Phase Analysis

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## Supporting Information

**ABSTRACT:** Burst-phase kinetic analysis was used to evaluate the deamination rate of the aminated–methylidene imidazolone (NH<sub>2</sub>–MIO) adduct of a *Taxus* phenylalanine aminomutase. The kinetic parameters were interrogated by a non-natural substrate (S)-styryl- $\alpha$ -alanine that yielded a chromophoric styrylacrylate product upon deamination by the aminomutase. Transient inactivation of the enzyme by the NH<sub>2</sub>–MIO adduct intermediate resulted in an initial burst of product, with reactivation by deamination of the adduct. This study validated the rate constants of a kinetic model demonstrating that the NH<sub>2</sub>–MIO adduct and cinnamate intermediate are sufficiently retained to catalyze the natural  $\alpha$ - to  $\beta$ -phenylalanine isomerization.

A subfamily of enzymes that includes aminomutases<sup>1–3</sup> and ammonia lyases<sup>4</sup> depends on the function of a 5-methylidene-1H-imidazol-5(4H)-one (MIO) cofactor within the active site. The recently solved structures of a phenylalanine aminomutases from *Pantoea agglomerans* (PaPAM)<sup>2</sup> and *Taxus canadensis* (TcPAM)<sup>1</sup> and a tyrosine aminomutase from *Streptomyces globisporus* (SgTAM)<sup>5</sup> support a mechanism where the amino group of the substrate attacks the MIO moiety (Scheme 1). The alkyl ammonium group is presumably

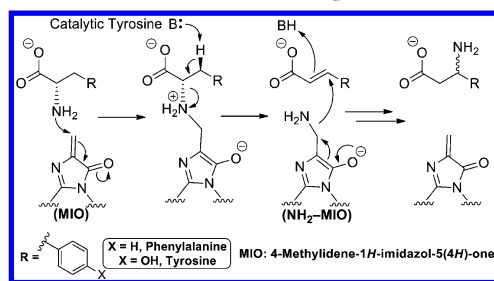
by C <sub>$\alpha$</sub>  to complete the isomerization. In the TcPAM reaction, the original stereochemical configuration at both C <sub>$\alpha$</sub>  and C <sub>$\beta$</sub>  is retained after the readdition of the NH<sub>2</sub> and proton. In contrast, the bacterial isozyme PaPAM and the related catalyst SgTAM invert the stereochemistry at the migration termini to make the corresponding  $\beta$ -amino acid product.<sup>5,6</sup>

The TcPAM reaction was deemed predominantly intramolecular; the amino group and proton from  $\alpha$ -phenylalanine rebound exclusively to the same carbon skeleton to make  $\beta$ -phenylalanine. Stable-isotope labeling studies revealed ~7% intermolecular amino group transfer from [<sup>15</sup>N]- $\alpha$ -phenylalanine to [<sup>2</sup>H<sub>6</sub>]-cinnamate (Scheme 2A).<sup>1</sup> This observation suggested the cinnamate intermediate occasionally diffused from the active site while the ammonia–enzyme (NH<sub>2</sub>–MIO; aminated 5-methylidene-1H-imidazol-5(4H)-one) (cf. Scheme 1) adduct remained intact. Thus, the lifetime of the NH<sub>2</sub>–MIO adduct was longer than the residence time of the cinnamate complex in the active site. Reciprocally, under the same reaction conditions, PaPAM did not transfer any label from [<sup>15</sup>N]- $\alpha$ -phenylalanine to [<sup>2</sup>H<sub>6</sub>]-cinnamate,<sup>6</sup> suggesting that the cinnamate remained in the active site longer than the lifetime of the NH<sub>2</sub>–MIO.

Another earlier study showed that during the SgTAM catalytic cycle, the transient amino group was transferred intermolecularly from 3'-chlorotyrosine to 4'-hydroxycinnamate (4'-HOCinn). This data suggested that the transient amino group remained attached to the enzyme during the course of the isomerization. This earlier study, however, did not evaluate an isotopically labeled-4'-HOCinn (an isotopomer of the natural pathway intermediate) to evaluate whether the amino group could transfer from  $\alpha$ -tyrosine to 4'-HOCinn. Thus, it was unclear if the pathway intermediate, after elimination of NH<sub>3</sub> from  $\alpha$ -tyrosine, could occasionally exchange intermolecularly with an exogenous source of 4'-HOCinn already in solution.<sup>7</sup>

An earlier study showed that TcPAM converted (S)-styryl- $\alpha$ -alanine to (2E,4E)-styrylacrylate (99%) (Scheme 2B) at approximately the same rate ( $k_{\text{cat}}$ ) as the natural (S)- $\alpha$ - to (R)- $\beta$ -phenylalanine isomerization. This suggested that during the conversion of styryl- $\alpha$ -alanine to styrylacrylate, the transient amino group likely remained as the NH<sub>2</sub>–MIO adduct for the same duration as the  $\alpha$ - to  $\beta$ -phenylalanine conversion. This hypothesis was interrogated by a mixture of (S)-styryl- $\alpha$ -alanine

**Scheme 1. Mechanism for a MIO-Dependent Aminomutase**



removed by an elimination (E2-like; E2 = bimolecular, concerted elimination reaction) mechanism that is initiated by removal of the *pro*-(3S) proton from the substrate by a catalytic tyrosine residue.<sup>1–3</sup> For TcPAM, the resulting cinnamate intermediate is principally trapped in the active site for the entire isomerization reaction<sup>1</sup> and rotates 180° about the C<sub>1</sub>–C <sub>$\alpha$</sub>  and C<sub>ipso</sub>–C <sub>$\beta$</sub>  bonds. The amino group of the aminated-MIO attacks C <sub>$\beta$</sub> , and the *pro*-(3S) proton is recovered

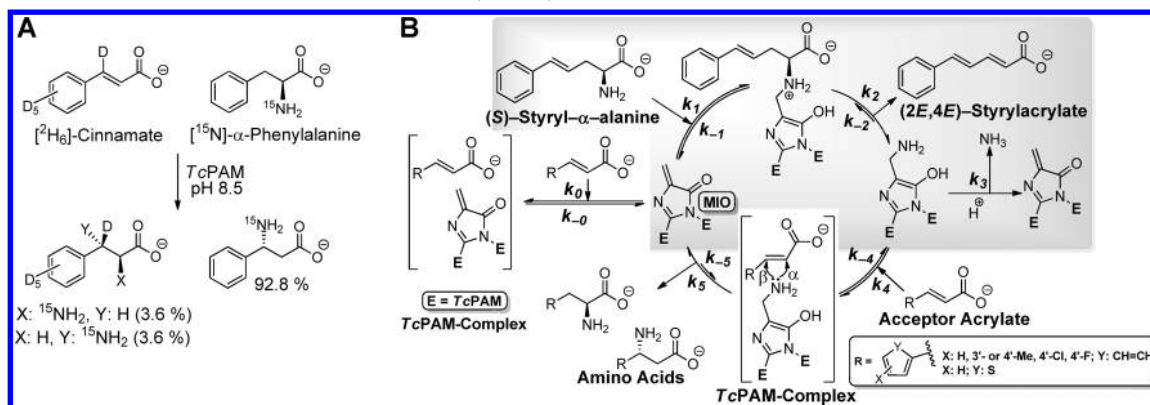
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Scheme 2. (A) Intermolecular Amino Group Transfer from [ $^{15}\text{N}$ ]- $\alpha$ -Phenylalanine to [ $^2\text{H}_6$ ]-Cinnamate by TcPAM Catalysis. (B) Kinetic Model for Transaminase Reaction Catalyzed by TcPAM<sup>a</sup>



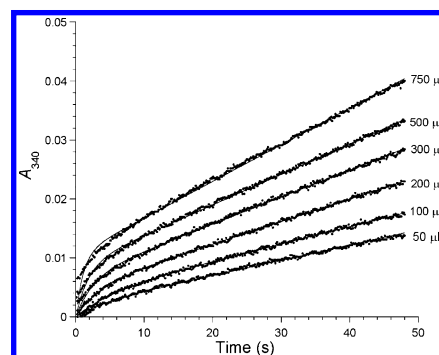
<sup>a</sup>Shaded inset: Kinetic model to evaluate the burst kinetics of the deamination of (S)-styryl- $\alpha$ -alanine.

(amino group donor) and an arylacrylate (amino group acceptor), both at steady state concentration. Acceptor substrates were selected, based on  $K_t$ , to estimate the relative binding affinities compared to that of (2E,4E)-styrylacrylate.<sup>8</sup>

The efficiency of the amino group transfer from the  $\text{NH}_2$ -MIO adduct in the described exchange reaction was largely dependent on the binding affinity of the acceptor acrylate for TcPAM. The amino group transferred at nearly 100% efficiency to a tighter-binding acceptor, while the efficiency decreased exponentially for weaker-binding acceptors when the  $\alpha$ - and  $\beta$ -amino acid mixtures were made.<sup>8</sup> This suggested that the transit time ( $1/k_4 + 1/k_5$ ) progressing from the amination of the acceptor to the release of the amino acids was significantly less than the transit time ( $1/k_3$ ) for the deamination of the  $\text{NH}_2$ -MIO adduct to the *apo*-MIO cofactor (Scheme 2B). Reciprocally, for the weakest binding acceptor, the transit times were exchanged, where  $(1/k_4 + 1/k_5) > (1/k_3)$ , and now the deamination of the  $\text{NH}_2$ -MIO adduct predominated. To further understand why the amine transfer efficiency decreased with weaker binding acceptors in the transamination reaction, and to infer the kinetics of the mechanistically similar MIO-dependent catalysts, the dissociation rate ( $k_3$ ) of the  $\text{NH}_2$ -MIO adduct in TcPAM was calculated herein.

Since TcPAM forms a transient covalent  $\text{NH}_2$ -MIO adduct in its interaction with amino acid substrates, a burst kinetic method was employed to establish the decay rate of the adduct. (S)-Styryl- $\alpha$ -alanine (Peptech Inc., Burlington, MA) was used as an adventitious substrate in the TcPAM-catalyzed reaction to show direct kinetic evidence of the accumulation of the purported ( $\text{NH}_2$ -MIO)-modified enzyme. The burst phase of TcPAM was evident in assays when (S)-styryl- $\alpha$ -alanine was converted to chromophoric product (2E,4E)-styrylacrylate in a fast step ( $k_2$ ), followed by slower release ( $k_3$ ) of the second product  $\text{NH}_3$  (Scheme 2B, shaded inset). The slower  $\text{NH}_3$ -release step led to the observed pre-steady state burst. In stopped-flow experiments, styrylacrylate was produced from styrylalanine initially along an exponential burst phase prior to reaching a steady-state progression (Figure 1). The burst phase parameters were then used to calculate the rate constants  $k_2$  and  $k_3$  (Scheme 2B).

Equation 1 is used generally to define the kinetic parameters when an enzyme-bound intermediate slowly dissociates to form the free enzyme.<sup>9</sup> This equation was applied to calculate the rate constants and burst phase parameters of the TcPAM



**Figure 1.** Evaluation of the kinetic model (Scheme 2B, shaded inset) for TcPAM burst kinetics. Equation 1 was used to globally fit experimental progress curves (Kaleidagraph 4.0) spanning six different (S)-styryl- $\alpha$ -alanine concentrations incubated with TcPAM (5.5  $\mu\text{M}$ ). Release of (2E,4E)-styrylacrylate was measured in a stopped-flow cell by  $A_{340}$  monitoring. Each time point is an average of three progression curves.

reaction. The formation of (2E,4E)-styrylacrylate was monitored at  $A_{340}$  ( $\epsilon_{340} = 4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ , pH 8.5) with a stopped flow spectrometer (model SX.18MV-R, dead-time = 2 ms, 1-cm optical path, thermostatically controlled at 23  $^\circ\text{C}$ ).

Aliquots (125  $\mu\text{L}$ ) of both TcPAM (5.5  $\mu\text{M}$  final concentration) and styryl- $\alpha$ -alanine (final concentrations between 50 and 750  $\mu\text{M}$ ) (Figure 1) were separately mixed, and the absorbance was measured at 0.12 s intervals over 50 s. The resulting data at each concentration were fit by nonlinear least-squares regression (eq 1) to obtain the burst amplitude (B) of the presteady-state and the velocity of the reaction at steady-state.

$$[P] = At + B(1 - e^{-k't}) \quad (1)$$

where the terms are  $[P]$ : product concentration; A: steady state velocity; B: burst amplitude;  $t$ : time; and  $k'$ : apparent first order rate constant of the presteady-state. Terms A and B are defined in the Supporting Information.

Terms  $A^2$  and B were simplified as a ratio and then rearranged (eq 2) to define a linear relationship between  $A^2$  and B at each substrate concentration. Since  $[E]_0$  was known,  $k_3$  was thus calculated from the slope ( $[E]_0 k_3^2$ ) of the plot of  $A^2$  against B (Figure 1S).

$$A^2 = B[E]_0 k_3^2 \quad (2)$$

The steady state kinetic parameters  $K_M^{\text{app}}$  and  $k_{\text{cat}}$  of TcPAM for styryl- $\alpha$ -alanine were calculated from a Hanes-Woolf plot of  $[S]_0/A$  against  $[S]_0$  (Figure 2S), where  $A$  was extracted from the steady-state formation of styrylacrylate (Figure 1, eq 1). Rate constant  $k_2$  was calculated from  $k_{\text{cat}}$  that relates  $k_3$  and  $k_2$  (eq 2S).

A linear relationship between  $A/B$  and  $1/[S]_0$  was established from eq 3 where  $k_3(k_3 + k_2)/k_2$  is the intercept (Figure 3S).

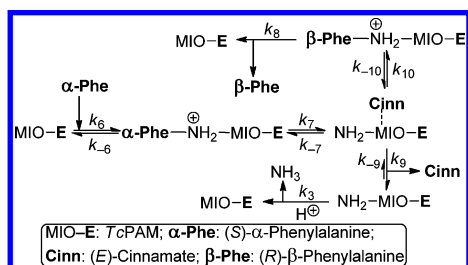
$$\frac{A}{B} = \frac{k_3(k_2 + k_3)}{k_2} + \frac{k_3(k_2 + k_3)K_M^{\text{app}}}{k_2} \frac{1}{[S]_0} \quad (3)$$

The experimentally determined values for  $k_2$  ( $0.19 \pm 0.01 \text{ s}^{-1}$ ) and  $k_3$  ( $0.041 \pm 0.002 \text{ s}^{-1}$ ) were substituted into  $k_3(k_3 + k_2)/k_2$ , and the resulting value ( $0.050 \pm 0.002 \text{ s}^{-1}$ ) was comparable to the intercept ( $0.049 \pm 0.004 \text{ s}^{-1}$ ) from eq 3. Thus, the burst phase kinetic analysis of styryl- $\alpha$ -alanine deamination by TcPAM was considered reliable.

The  $K_M^{\text{app}}$  of TcPAM for styryl- $\alpha$ -alanine was  $105 \pm 10 \mu\text{M}$  and the  $k_{\text{cat}}$  was  $0.034 \pm 0.002 \text{ s}^{-1}$  at  $23^\circ\text{C}$  (pH 8.5). These values were agreeable ( $K_M^{\text{app}} = 250 \mu\text{M}$ ;  $k_{\text{cat}} = 0.082 \text{ s}^{-1}$ ) to those reported in an earlier study for the same reaction, at  $31^\circ\text{C}$ .<sup>8</sup> The  $K_M$  ( $588 \pm 37 \mu\text{M}$ ) of TcPAM was calculated from  $K_M^{\text{app}}$  (see eq 4S) for styryl- $\alpha$ -alanine in the ammonia elimination reaction that produced styrylacrylate (Scheme 2B, shaded inset). The burst amplitude ( $B$ ) and steady-state velocity ( $A$ ) was dependent on  $[S]_0$  (Figure 1). The rate constant ( $k_3 = 0.041 \pm 0.002 \text{ s}^{-1}$ ) for the release of  $\text{NH}_3$  (that reset TcPAM for another catalytic cycle) was similar to  $k_{\text{cat}}$  ( $0.034 \pm 0.002 \text{ s}^{-1}$ ) for the conversion of styryl- $\alpha$ -alanine to styrylacrylate. In contrast, the calculated rate constant ( $k_2 = 0.19 \pm 0.01 \text{ s}^{-1}$ ) for the release of styrylacrylate was 5-fold greater than  $k_{\text{cat}}$ . Therefore,  $k_{\text{cat}}$  for the overall reaction was slowed 5-fold likely by the slower deamination rate of enzyme.

TcPAM normally deaminates  $\alpha$ -phenylalanine in the first committed step, and the ensuing cinnamate complex, the protonated catalytic residue, and the  $\text{NH}_2$ -MIO adduct (see Scheme 1) are retained long enough to complete the intramolecular isomerization.<sup>1</sup> Under steady-state conditions, the  $k_{\text{cat}}^{\alpha/\beta}$  for this conversion was  $0.014 \pm 0.001 \text{ s}^{-1}$  at  $23^\circ\text{C}$ , corresponding to a transit time ( $1/k_{\text{cat}}^{\alpha/\beta}$ ) of 71 s. In addition,  $\beta$ -phenylalanine and cinnamate were produced at a 9:1 ratio. This kinetics data suggested that the transit time ( $1/k_7 + 1/k_9 + 1/k_3$ ) from the  $\alpha\text{-Phe-NH}_2\text{-MIO-E}$  complex to the release of  $\text{NH}_3$ , via the cinnamate-production pathway (Scheme 3), was  $\sim 9$  times longer (639 s) than  $1/k_{\text{cat}}^{\alpha/\beta}$ . Since  $1/k_3$  (24 s) on this pathway is known, then  $(1/k_7 + 1/k_9)$ , the estimated transit time to cinnamate (Scheme 3), was therefore 615 s. The latter transit time supported the notion that the dwell time of

**Scheme 3. Kinetic Model for the Proposed Mechanism of the TcPAM-Catalyzed Conversion of  $\alpha$ - to  $\beta$ -Phenylalanine**



cinnamate in the active site is sufficient to preferentially promote the intramolecular amino group rebound pathway to  $\beta$ -phenylalanine.

In conclusion, TcPAM undergoes finite inactivation during turnover of (S)-styryl- $\alpha$ -alanine to styrylacrylate, resulting in burst kinetics with the steady-state rate being a dynamic balance between the inactive modified enzyme ( $\text{NH}_2$ -MIO adduct) and reactivation by deamination of the adduct. To our knowledge there are no reports on the direct kinetic evaluation of this deamination for any MIO-dependent enzyme. In this study, the calculated deamination rate constant for the dissociation of the  $\text{NH}_2$ -MIO adduct contributes information to further understand the mechanism of the TcPAM reaction.

## ■ ASSOCIATED CONTENT

### Supporting Information

Linear regression analyses of steady state parameters, chemical suppliers, enzyme preparation, Michaelis constant and burst phase equations, and quantification of biosynthetic styrylacrylate. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## ■ AUTHOR INFORMATION

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### Notes

The authors declare no competing financial interest.

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