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

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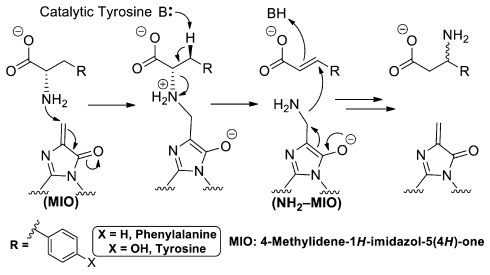
Supporting Information

ABSTRACT: Burst-phase kinetic analysis was used to evaluate the deamination rate of the aminated−methylidene imidazolone (NH_2−MIO) adduct of a *Taxus* phenylalanine aminomutase. The kinetic parameters were interrogated by a non-natural substrate (*S*)-styryl-α-alanine that yielded a chromophoric styrylacrylate product upon deamination by the aminomutase. Transient inactivation of the enzyme by the NH_2−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_2−MIO adduct and cinnamate intermediate are sufficiently retained to catalyze the natural α- to β-phenylalanine isomerization.

A subfamily of enzymes that includes aminomutases[1−3] and ammonia lyases[4] depends on the function of a 5-methylidene-1*H*-imidazol-5(4*H*)-one (MIO) cofactor within the active site. The recently solved structures of a phenylalanine aminomutases from *Pantoea agglomerans* (*Pa*PAM)[2] and *Taxus canadensis* (*Tc*PAM)[1] and a tyrosine aminomutase from *Streptomyces globisporus* (*Sg*TAM) [5] support a mechanism where the amino group of the substrates attacks the MIO moiety (Scheme 1). The alkyl ammonium group is presumably

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*-(3*S*) proton from the substrate by a catalytic tyrosine residue.[1−3] For *Tc*PAM, the resulting cinnamate intermediate is principally trapped in the active site for the entire isomerization reaction[1] and rotates 180° about the C_1−C_α and C_{ipso}−C_β bonds. The amino group of the aminated-MIO attacks C_β, and the *pro*-(3*S*) proton is recovered by C_α to complete the isomerization. In the *Tc*PAM reaction, the original stereochemical configuration at both C_α and C_β is retained after the readdition of the NH_2 and proton. In contrast, the bacterial isozyme *Pa*PAM and the related catalyst *Sg*TAM invert the stereochemistry at the migration termini to make the corresponding β-amino acid product.[5,6]

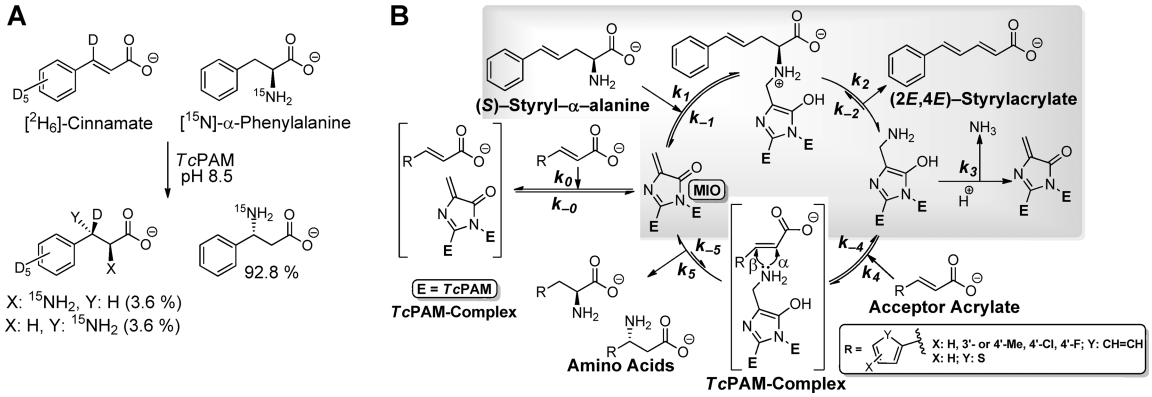
The *Tc*PAM reaction was deemed predominantly intramolecular; the amino group and proton from α-phenylalanine rebound exclusively to the same carbon skeleton to make β-phenylalanine. Stable-isotope labeling studies revealed ∼7% intermolecular amino group transfer from [^{15}N]-α-phenylalanine to [$^2H_6$]-cinnamate (Scheme 2A).[1] This observation suggested the cinnamate intermediate occasionally diffused from the active site while the ammonia−enzyme (NH_2−MIO; aminated 5-methylidene-1H-imidazol-5(4H)-one) (cf. Scheme 1) adduct remained intact. Thus, the lifetime of the NH_2−MIO adduct was longer than the residence time of the cinnamate complex in the active site. Reciprocally, under the same reaction conditions, *Pa*PAM did not transfer any label from [^{15}N]-α-phenylalanine to [$^2H_6$]-cinnamate,[6] suggesting that the cinnamate remained in the active site longer than the lifetime of the NH_2−MIO.

Another earlier study showed that during the *Sg*TAM 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 α-tyrosine to 4′-HOCinn. Thus, it was unclear if the pathway intermediate, after elimination of NH_3 from α-tyrosine, could occasionally exchange intermolecularly with an exogenous source of 4′-HOCinn already in solution.[7]

An earlier study showed that *Tc*PAM converted (*S*)-styryl-α-alanine to (2*E*,4*E*)-styrylacrylate (99%) (Scheme 2B) at approximately the same rate (k_{cat}) as the natural (*S*)-α- to (*R*)-β-phenylalanine isomerization.[1] This suggested that during the conversion of styryl-α-alanine to styrylacrylate, the transient amino group likely remained as the NH_2−MIO adduct for the same duration as the α- to β-phenylalanine conversion. This hypothesis was interrogated by a mixture of (*S*)-styryl-α-alanine

Received: May 1, 2012
Revised: May 30, 2012
Published: June 11, 2012

Scheme 2. (A) Intermolecular Amino Group Transfer from [^{15}N]-α-Phenylalanine to [$^{2}H_6$]-Cinnamate by *Tc*PAM Catalysis. (B) Kinetic Model for Transaminase Reaction Catalyzed by *Tc*PAM[a]



[a]Shaded inset: Kinetic model to evaluate the burst kinetics of the deamination of (*S*)-styryl-α-alanine.

(amino group donor) and an arylacrylate (amino group acceptor), both at steady state concentration. Acceptor substrates were selected, based on K_I, to estimate the relative binding affinities compared to that of (2*E*,4*E*)-styrylacrylate.[8]

The efficiency of the amino group transfer from the NH_2−MIO adduct in the described exchange reaction was largely dependent on the binding affinity of the acceptor acrylate for *Tc*PAM. The amino group transferred at nearly 100% efficiency to a tighter-binding acceptor, while the efficiency decreased exponentially for weaker-binding acceptors when the α- and β-amino acid mixtures were made.[8] 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 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 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 NH_2−MIO adduct in *Tc*PAM was calculated herein.

Since *Tc*PAM forms a transient covalent 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-α-alanine (Peptech Inc., Burlington, MA) was used as an adventitious substrate in the *Tc*PAM-catalyzed reaction to show direct kinetic evidence of the accumulation of the purported (NH_2−MIO)-modified enzyme. The burst phase of *Tc*PAM was evident in assays when (*S*)-styryl-α-alanine was converted to chromophoric product (2*E*,4*E*)-styrylacrylate in a fast step (k_2), followed by slower release (k_3) of the second product NH_3 (Scheme 2B, shaded inset). The slower 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.[9] This equation was applied to calculate the rate constants and burst phase parameters of the *Tc*PAM

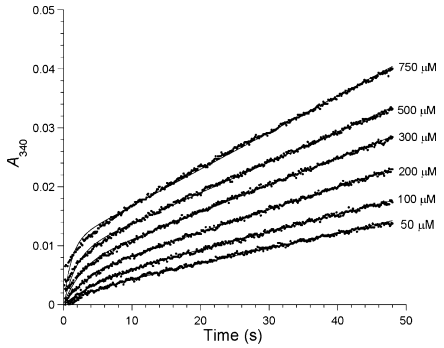


Figure 1. Evaluation of the kinetic model (Scheme 2B, shaded inset) for *Tc*PAM burst kinetics. Equation 1 was used to globally fit experimental progress curves (Kaleidagraph 4.0) spanning six different (*S*)-styryl-α-alanine concentrations incubated with *Tc*PAM (5.5 μM). Release of (2*E*,4*E*)-styrylacrylate 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 (2*E*,4*E*)-styrylacrylate was monitored at A_{340} ($\varepsilon_{340} = 4 \times 10^3$ M^{-1} 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 °C).

Aliquots (125 μL) of both *Tc*PAM (5.5 μM final concentration) and styryl-α-alanine (final concentrations between 50 and 750 μM) (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 $[E]_o$ was known, k_3 was thus calculated from the slope $([E]_o k_3^2)$ of the plot of A^2 against *B* (Figure 1S).

dx.doi.org/10.1021/bi300569r | *Biochemistry* 2012, 51, 5226−5228

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

The steady state kinetic parameters K_M^{app} and k_{cat} of *Tc*PAM for styryl-α-alanine were calculated from a Hanes-Woolf plot of $[S]_o/A$ against $[S]_o$ (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_{cat} that relates k_3 and k_2 (eq 2S).

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

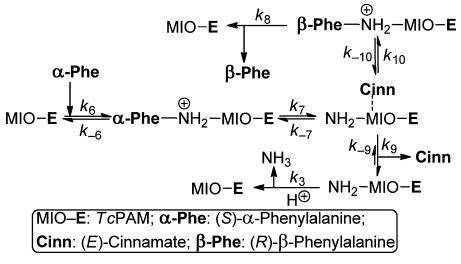
$$\frac{A}{B} = \frac{k_3(k_2 + k_3)}{k_2} + \frac{k_3(k_2 + k_3)K_M^{app}}{k_2}\frac{1}{[S]_o} \quad (3)$$

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

The K_M^{app} of *Tc*PAM for styryl-α-alanine was 105 ± 10 μM and the k_{cat} was 0.034 ± 0.002 s^{-1} at 23 °C (pH 8.5). These values were agreeable (K_M^{app} = 250 μM; k_{cat} = 0.082 s^{-1}) to those reported in an earlier study for the same reaction, at 31 °C.[8] The K_M (588 ± 37 μM) of *Tc*PAM was calculated from K_M^{app} (see eq 4S) for styryl-α-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 $[S]_o$ (Figure 1). The rate constant (k_3 = 0.041 ± 0.002 s^{-1}) for the release of NH_3 (that reset *Tc*PAM for another catalytic cycle) was similar to k_{cat} (0.034 ± 0.002 s^{-1}) for the conversion of styryl-α-alanine to styrylacrylate. In contrast, the calculated rate constant (k_2 = 0.19 ± 0.01 s^{-1}) for the release of styrylacrylate was 5-fold greater than k_{cat}. Therefore, k_{cat} for the overall reaction was slowed 5-fold likely by the slower deamination rate of enzyme.

*Tc*PAM normally deaminates α-phenylalanine in the first committed step, and the ensuing cinnamate complex, the protonated catalytic residue, and the NH_2−MIO adduct (see Scheme 1) are retained long enough to complete the intramolecular isomerization.[1] Under steady-state conditions, the $k_{cat}^{\alpha/\beta}$ for this conversion was 0.014 ± 0.001 s^{-1} at 23 °C, corresponding to a transit time ($1/k_{cat}^{\alpha/\beta}$) of 71 s. In addition, β-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 α-Phe−$^+NH_2$−MIO−E complex to the release of NH_3, via the cinnamate-production pathway (Scheme 3), was ~9 times longer (639 s) than $1/k_{cat}^{\alpha/\beta}$. 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 cinnamate in the active site is sufficient to preferentially promote the intramolecular amino group rebound pathway to β-phenylalanine.

Scheme 3. Kinetic Model for the Proposed Mechanism of the *Tc*PAM-Catalyzed Conversion of α- to β-Phenylalanine



In conclusion, *Tc*PAM undergoes finite inactivation during turnover of (*S*)-styryl-α-alanine to styrylacrylate, resulting in burst kinetics with the steady-state rate being a dynamic balance between the inactive modified enzyme (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 NH_2−MIO adduct contributes information to further understand the mechanism of the *Tc*PAM 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.

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Funding

Funding by National Science Foundation (CAREER Award 0746432 to K.D.W.).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge Prof. Hanggao Yan, Department of Biochemistry, Michigan State University for providing the stopped-flow spectrometer.

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