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Detection of Transient Intermediates in the Metal-Dependent Nonoxidative Decarboxylation Catalyzed by α -Amino- β -Carboxymuconate- ϵ -Semiaidehyde Decarboxylase

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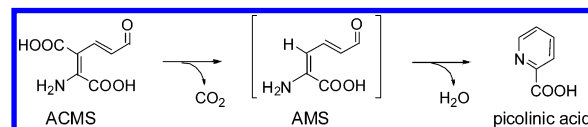
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The first description of a metalloenzyme-mediated nonoxidative decarboxylation reaction was that catalyzed by α -amino- β -carboxymuconate- ϵ -semialdehyde decarboxylase (ACMSD) (Scheme 1).^{1,2} ACMSD is an important enzyme in tryptophan metabolism and nitroaromatic biodegradation.^{2–4} In the absence of ACMSD, the substrate α -amino- β -carboxymuconate- ϵ -semialdehyde (ACMS) is nonenzymatically converted to quinolinic acid, a neurotoxin.^{5–7} Analysis of the crystal structure of ACMSD, along with EPR spectroscopy of the enzyme, has revealed that the active site contains a mononuclear metal cofactor.^{2,8,9} ACMSD retains activity with several different divalent metal ions at this site, including zinc, cobalt, iron, and manganese.^{2,8,9} Recent studies have shown that several other decarboxylases, including the structurally characterized enzyme γ -resorcyolate decarboxylase, utilize a d-block metal ion to catalyze a variety of nonoxidative decarboxylation reactions.^{1,10} These enzymes catalyze similar chemical reactions, share similar overall and active site structures, and belong to the same subset of enzymes in the amidohydrolase superfamily.^{11,12} Within this large enzyme superfamily, most members catalyze hydrolytic reactions. The newly defined ACMSD protein subgroup is unique in that its members catalyze nonhydrolytic C–C bond cleavage.^{1,9} Thus far, the catalytic mechanism of this novel type of metallocofactor-dependent nonoxidative decarboxylation has remained largely uncharacterized.

In this work, we conducted a pre-steady-state kinetic study of the reaction catalyzed by ACMSD isolated from *Pseudomonas fluorescens*. These studies take advantage of the fact that the substrate, ACMS, is a chromophore ($\epsilon_{360\text{ nm}} = 47\,500\text{ M}^{-1}\text{ cm}^{-1}$), while the final product, picolinic acid, is transparent at 300 nm or above.¹³ ACMSD may be reconstituted after purification with different metal ions. Of these, Co(II)-reconstituted ACMSD exhibits the highest activity and the greatest stability, which facilitated kinetic analysis of this enzyme.² Transient kinetic experiments were conducted in 25 mM HEPES buffer, pH 7.0, 5% glycerol at 25 °C using an OLIS rapid scanning stopped-flow spectrophotometer. Pseudo-first-order conditions were maintained using 1 μM ACMS as the limiting reagent, while the concentration of Co(II)-reconstituted ACMSD was varied from 7.5 to 45 μM . Protein concentration was initially estimated using the Coomassie Plus (Pierce) assay, and then the precise amount of metal-reconstituted ACMSD was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES).

The ACMSD reaction was clearly multiphasic, exhibiting a rapid rate which was dependent on ACMSD concentration and two slower rates which were independent of ACMSD concentration (Figure 1). A species (intermediate I) with an absorption maximum red-shifted relative to that of the substrate is rapidly formed concomitant with the loss of ACMS absorbance centered at 360 nm (Figure 2). This species is then converted in a slower reaction to a species (intermediate II) with a similar absorption maximum but a

Scheme 1



decreased extinction coefficient. Complete formation of intermediate II, with the loss of substrate and intermediate I, occurs within 1 s (Figure 2B). The primary data on which Figure 2 is based are shown in Figure S1. Conversion of intermediate II to the final colorless product, picolinic acid, requires several additional seconds (not shown). Since intermediate II forms quantitatively, it was possible to calculate its $\epsilon_{383} = 40\,000\text{ cm}^{-1}\text{ M}^{-1}$ by comparison with that of ACMS ($\epsilon_{360} = 47\,500\text{ cm}^{-1}\text{ M}^{-1}$). It was not possible to accurately determine this value for intermediate I because it does not form quantitatively, as it is dependent upon the relative rates of formation and decay and thus will vary with [ACMSD] used.

The chemical nature of intermediate I is proposed to be α -aminomuconate- ϵ -semialdehyde (AMS), a previously proposed but undetected decarboxylation intermediate of the reaction¹⁴ in complex with ACMSD (Scheme 2). The observed rate for the conversion of ACMS to intermediate I exhibited a linear dependence on the concentration of ACMSD (Figure 1). The observation of a linear rather than hyperbolic concentration dependence indicates that the formation of the enzyme–substrate complex is slow relative to the catalytic step. The value of k_1 in Scheme 2 determined from the slope of the line is $2.4 \times 10^6\text{ s}^{-1}\text{ M}^{-1}$. The second kinetic phase is concentration independent and therefore a unimolecular reaction. This reaction, which exhibits a rate of $8.8 \pm 1.0\text{ s}^{-1}$, is proposed to be the release of AMS from the enzyme–product complex (k_3 in Scheme 2). This is assumed to be a virtually irreversible reaction. This would be consistent with the similar spectral properties of intermediates I and II, with I being AMS bound to the enzyme and II being free AMS. The rate for the disappearance of intermediate II (k_4) is $0.05 \pm 0.02\text{ s}^{-1}$ and is also independent of

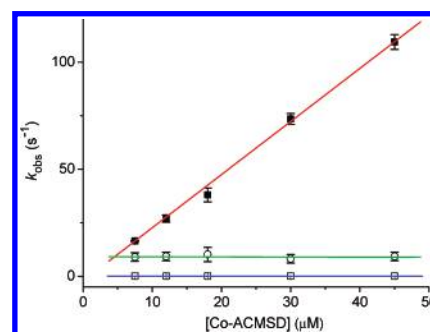
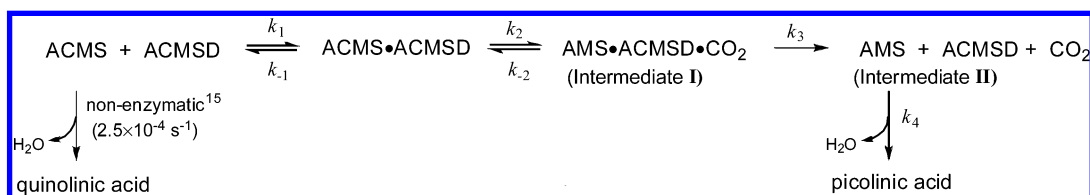


Figure 1. Concentration dependence of the observed rates for the formation of intermediate I (red, ■), conversion of intermediate I to intermediate II (green, ○), and disappearance of intermediate II (blue, □).

Scheme 2



ACMSD concentration, consistent with it describing the spontaneous cyclization of released AMS to picolinic acid. Since a hyperbolic dependence of the reaction rate with enzyme concentration was not observed, it was not possible to determine k_2 (the rate constant for the catalytic conversion of ACMS to AMS). It can be concluded that the rate of the catalytic conversion of ACMS to AMS is rapid relative to formation of the enzyme–substrate complex (i.e., $k_2 > k_1[\text{ACMSD}] + k_{-1}$) and release of AMS from the enzyme–product complex (i.e., $k_2 > k_3$).

The rate of product release ($k_3 = 8.8 \text{ s}^{-1}$) is approximately the same as k_{cat} for the steady-state reaction of Co(II)–ACMSD with ACMS, which was measured under the same reaction conditions, as 7.3 s^{-1} .² It should be noted that in those steady-state studies the reaction was monitored by the initial rate of the loss of absorbance at 360 nm. As can be seen in Figure 2, the reaction monitored at this wavelength describes the steady-state conversion of ACMS to AMS, not to the colorless picolinic acid. This explains why the observed steady-state k_{cat} was similar to k_3 rather than the slower k_4 .

Similar experiments were performed using Mn(II)-reconstituted ACMSD, and the accumulation of intermediates with absorption

spectra identical to those shown in Figure 2 was observed (data not shown). This suggests that the nature of the metal at the active site does not affect the chemical reaction mechanism of ACMSD. Unfortunately, instability of Mn(II)–ACMSD, along with incomplete reconstitution of the metal, prevented accurate determination of the concentration of active enzyme so that a kinetic analysis like that described here for Co(II)–ACMSD was not possible.

The reaction catalyzed by ACMSD is biologically significant because this enzyme of the tryptophan degradation pathway converts a catabolic intermediate to a benign metabolite, thus preventing the accumulation of a neurotoxic compound. The conversion of ACMS substrate to picolinic acid requires both a decarboxylation and a cyclization reaction. These results show that the enzyme-catalyzed decarboxylation occurs first to generate an unstable AMS intermediate, which then undergoes a relatively slow release from the enzyme and a much slower cyclization to yield the final product. Previous steady-state kinetic studies indicated that the identity of the bound metal of ACMSD affected enzyme stability and K_m but had relatively little effect on k_{cat} .² The latter effect may be explained by the observation that the rate-limiting step is not the metal-dependent decarboxylation but the subsequent slower reaction. This information provides new insight into our understanding of the chemical and kinetic reaction mechanisms of this recently characterized metal-ion-mediated nonoxidative decarboxylation.

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Supporting Information Available: Figure S1 displays the original time courses of spectral scans from which Figure 2 is derived. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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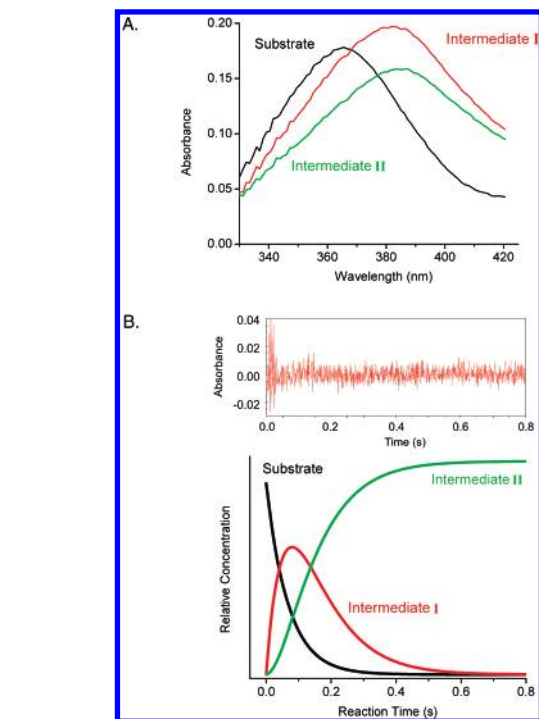


Figure 2. Results of the reaction of 1 μM ACMS with 12 μM ACMSD. Reconstructed spectra (A) and changes in concentration with time (B) of the kinetically distinguishable species fit to an $A \rightarrow B \rightarrow C$ model with the OLIS GlobalWorks software. The plot of the residuals for the fit of data is shown as an inset at the top.