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Isochorismate Pyruvate Lyase: A Pericyclic Reaction Mechanism?

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Despite their broad utility in the laboratory, pericyclic reactions are rarely exploited in cellular metabolism. The Claisen rearrangement of chorismate to prephenate,[1] the suprafacial [1,5]-sigmatropic shift of a methyl group in the transformation of precorrin-8x to hydrogenobyrinic acid,[2] and several putative Diels−Alder reactions[3] are the only processes for which actual enzyme catalysts have been identified. Here we present evidence that isochorismate pyruvate lyase (IPL) from *Pseudomonas aeruginosa*,[4,5] an enzyme involved in bacterial siderophore biosynthesis, should be added to this short list.

IPL catalyzes the elimination of the enolpyruvyl side chain from isochorismate to give salicylate and pyruvate (Scheme 1a). This type of aromatization reaction is generally formulated as a base-initiated process (Scheme 1b),[6] but a dissociative mechanism involving initial cleavage of the C−O bond to give an ion pair intermediate is conceivable (Scheme 1c). A concerted pericyclic pathway, in which the hydrogen atom at C2 is transferred to C9 of the side chain simultaneous with C−O cleavage (Scheme 1d), which represents a third possibility,[7] has been proposed as well.[8]

We employed isochorismate selectively deuterated at C2 to distinguish between these mechanistic alternatives. The labeled substrate was prepared chemoenzymatically from D-mannose. Briefly, the sugar was converted to sodium [6,6-$^2H_2$]shikimate in 12 steps following a modified procedure for preparing unlabeled shikimic acid.[9] Incubation of labeled shikimate with phosphoenolpyruvate and cell extracts from an engineered *Escherichia coli* strain (KA12/pKAD50/pKS3-02),[10] which lacks chorismate mutase but overproduces the enzymes shikimate kinase, EPSP synthase, chorismate synthase, and isochorismate synthase, afforded [2-^{2}H]-isochorismate in ca. 10% yield. The latter was isolated by extraction and purified by preparative reverse phase HPLC.[10]

P. aeruginosa IPL cleanly converts isochorismate to salicylate and pyruvate as verified both by ^{1}H NMR and HPLC analysis of typical reaction mixtures. The steady-state kinetic parameters obtained with the unlabeled substrate and [2-^{2}H]isochorismate in 50 mM phosphate buffer (pH 7.5) at 30 °C are reported in Table 1. On the basis of these data, the ^{2}H kinetic isotope effects on k_{cat} and k_{cat}/K_m are 2.34 ± 0.08 and 1.75 ± 0.18, respectively. The observed effect on k_{cat} establishes that chemistry is significantly rate determining for the enzyme. Moreover, the magnitude of the isotope effect is consistent with considerable C−H bond cleavage in the transition state (Scheme 1b or 1d), but would be unprecedentedly large for a β-secondary effect in an ion pair mechanism (Scheme 1c).[11] The somewhat smaller isotope effect observed on k_{cat}/K_m indicates a significant forward commitment to catalysis for IPL.[12]

The fate of the deuterium atom at C2 was determined directly by ^{2}H NMR spectroscopy. The spectrum of [2-^{2}H]isochorismate in buffer containing dioxane *d*-8 as an internal standard was recorded in the absence of enzyme (Figure 1a). After addition of IPL, the sample was incubated at 30 °C for 30 min. During the ensuing reaction, the deuterium label was quantitatively transferred to pyruvate (Figure 1b). The signals for substrate prior to reaction and [3-^{2}H]pyruvate upon completion of the reaction gave identical integrations within experimental error (4.66 ± 0.32 mM versus 4.74 ± 0.32 mM, respectively). In contrast to the uncatalyzed reaction at 60 °C, where significant exchange of the labeled pyruvate with solvent complicates analysis,[10] no exchange was observed over several hours at the lower temperature of the enzymatic assay; the small amount of HOD seen at 4.55 ppm in the spectrum of the reaction mixture (Figure 1b) derives from the added enzyme stock solution, which was not prepared from deuterium-depleted H_2O.

The finding that the deuterium originally at C2 of isochorismate is quantitatively transferred to pyruvate in the IPL-catalyzed elimination reaction argues strongly against a mechanism requiring an external base (Scheme 1b). The failure to identify an active site residue in IPL by mutagenesis and genetic selection that could plausibly serve as such a base[8] further strengthens this conclusion.

A significant ^{2}H kinetic isotope effect and quantitative transfer of the label to pyruvate are both consistent with a pericyclic reaction mechanism (Scheme 1d). Hybrid density functional theory (HDFT) calculations[13] also support this possibility. A low energy transition structure for a concerted but asynchronous [1,5]-sigmatropic shift, in which carbon−oxygen bond cleavage is more advanced than hydrogen atom transfer from C2 to C9, was located at the Becke3LYP/DZ+(2d,p) level of theory (Figure 2). This structure,

Scheme 1

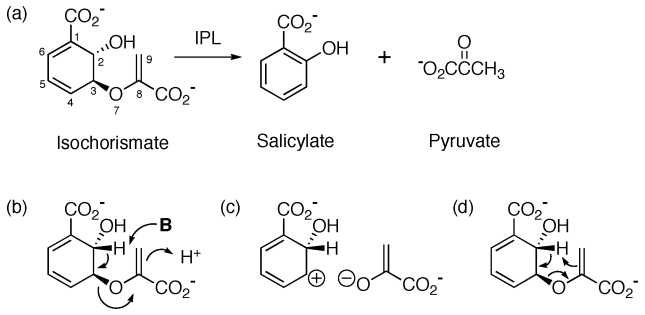

Table 1. Kinetic Parameters for *P. aeruginosa* Isochorismate Pyruvate Lyase[a]

substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
isochorismate	1.01 ± 0.02	1.05 ± 0.08	(9.62 ± 0.73) × 10^5
[2-^{2}H]-isochorismate	0.43 ± 0.01	0.79 ± 0.05	(5.49 ± 0.37) × 10^5

[a] Enzyme was isolated as described in ref 8. Reactions were performed in triplicate in 50 mM phosphate buffer (pH 7.5, 30 °C). Kinetic parameters for the unlabeled substrate are in excellent agreement with previous data (k_{cat} = 1.06 s^{-1}, K_m = 0.95 μM).[8]

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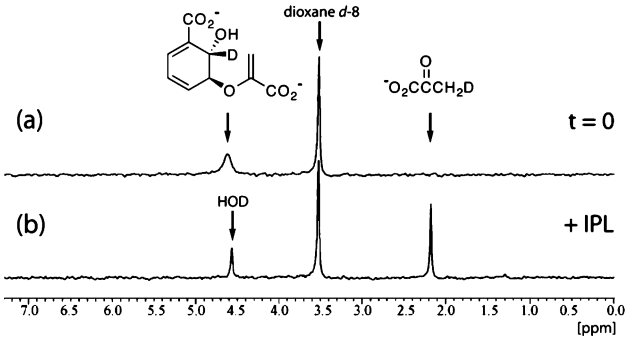

Figure 1. (a) ^{2}H NMR spectrum of [2-^{2}H]isochorismate in 50 mM phosphate buffer (pH 7.5) at 30 °C using deuterium-depleted H_2O and 1.00 mM dioxane *d*-8 as internal standard. (b) ^{2}H NMR spectrum of labeled isochorismate (a) after incubation with 25 μM IPL (in natural abundance H_2O, hence the small peak at 4.55 ppm) for 30 min at 30 °C.

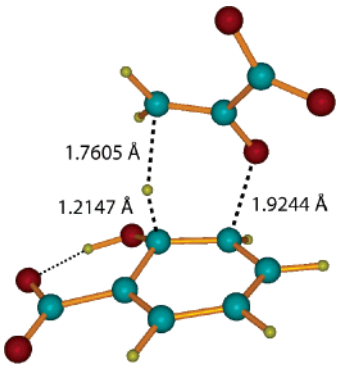

Figure 2. Becke3LYP/DZ+(2d,p) structure of the pericyclic transition state for the conversion of isochorismate to salicylate and pyruvate.

which can be directly accessed from the predominant pseudodiequatorial substrate conformer, predicts an isotope effect of 2.222 for the elimination reaction, in good agreement with the observed effect on k_{cat}. The isotopically sensitive bond-breaking step in the enzymatic reaction thus appears to be fully rate determining in IPL.

In summary, experiment and computation point to a one-step pericyclic mechanism for the IPL-catalyzed conversion of isochorismate to salicylate and pyruvate. Expansion of the small set of enzymes known to catalyze sigmatropic rearrangements promises to enhance our understanding of the strategies proteins use to accelerate this fundamental class of reaction.[14] Additionally, our finding raises the intriguing possibility that other protein catalysts will be found for such processes. The IPL transformation is formally similar to the elimination of pyruvate from other shikimate metabolites, including chorismate, 4-amino-4-deoxychorismate, and 2-amino-2-deoxyisochorismate, catalyzed by chorismate lyase[15] or salicylate synthase,[16] 4-amino-4-deoxychorismate lyase,[17] and anthranilate synthase,[18] respectively. The importance of these enzymes for the production of diverse aromatic metabolites, including ubiquinones, siderophores, folates, and amino acids,[6] warrants careful reinvestigation of their mechanism of action.

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Supporting Information Available: Coordinates and computational details for calculation of the theoretical isotope effects, and complete ref 13b. This material is available free of charge via the Internet at http://pubs.acs.org.

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