Communications to the Editor

Absolute Stereochemical Course of Muconolactone Δ-Isomerase and of 4-Carboxymuconolactone Decarboxylase: A ¹H NMR "Ricochet" Analysis

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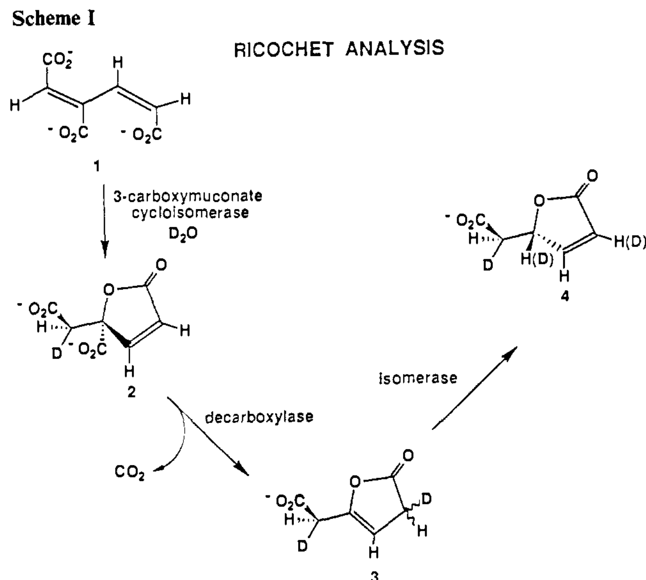
Muconolactone Δ-isomerase [EC 5.3.3.4; 2-oxo-2,3-dihydrofuran-5-acetate Δ^3-Δ^4-isomerase] and 4-carboxymuconolactone decarboxylase [EC 4.1.1.44; 4-carboxymuconolactone carboxy-lyase] from *Pseudomonas putida* and from *Acinetobacter calcoaceticus* generate a common product on the β-ketoadipate pathway—β-ketoadipate enol-lactone (Scheme I).[1] In light of the configurational assignments for muconolactone and 4-carboxymuconolactone discussed in the preceeding paper,[2] the stereochemical course of both enzymatic reactions is of considerable interest. Two observations pose potential complications to the analysis: the α-hydrogens of the enol-lactone are exchangeable resulting in a loss of stereochemistry at this position,[3] and the equilibrium constant for the muconolactone to enol-lactone isomerization favors the former by a factor of ~13.[4] We report herein the results of a ¹H NMR "ricochet" experiment which utilizes these observations to permit the simultaneous and unequivocal stereochemical analysis of both enzymatic reactions.

The basis of the experiment is outlined in Scheme I. In ^2H_2O, 3-carboxy-*cis,cis*-muconate (**1**) is converted to (4*R*,5*R*)-5-[²H]-4-carboxymuconolactone (**2**) by the action of 3-carboxymuconate cycloisomerase from *P. putida*.[2] Enzymatic decarboxylation yields a dideuteriated enol-lactone (**3**) which retains the R configuration at C-5 and is presumably chirally deuteriated at C-2. This enol-lactone is "ricocheted" to a deuteriated (4*S*)-muconolactone **4** taking advantage of the favorable equilibrium constant.[5] Rapid ¹H NMR analysis of the muconolactone is used to determine the fate of the initial protons and the positions of deuterium incorporation. A number of possible scenarios can be envisioned; the actual result afforded the only unambiguous sequence of events.

3-Carboxy-*cis,cis*-muconate in the presence of the isomerase and decarboxylase in ^2H_2O affords a simple NMR spectrum (Figure 1a).[6] Initiation of the reaction with the cycloisomerase

Scheme I



rapidly generates a complex spectrum (Figure 1b) indicative of the four compounds present at this stage.[7] After 15 min, simplicity returns with the near quantitative formation of (4*S*,5*R*)-2,5-di[²H]muconolactone (Figure 1c).[8]

The key to the interpretation of this experiment is the unambiguous appearance at C-4 of muconolactone of a proton (5.32 ppm) which in ^2H_2O could only be derived by its direct transfer from C-2 of the enol-lactone (Scheme II). This reasonably infers a suprafacial proton transfer by a single univalent base. Muconolactone isomerase, therefore, proceeds by a syn mechanism, and the transfer of the proton and not the deuteron from C-2 of the enol-lactone assigns the R configuration to C-2. Finally, the enzymatic decarboxylation of (4*R*,5*R*)-5-[²H]-4-carboxymuconolactone to (2*R*,5*R*)-2,5-di[²H]enol-lactone is thus established as a syn process.

The structural similarities and amino acid sequence homology of the isomerase and the decarboxylase have led to the hypothesis that these distinct enzymes may have evolved from an ancestral

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(1) (a) Stanier, R. Y.; Ornston, L. N. In *Advances in Microbial Physiology*; Rose, A. H., Tempest, D. W., Eds.; Academic Press: London and New York; 1973; Vol. 9, pp 89-151. (b) Parke, D.; Meagher, R. B.; Ornston, L. N. *Biochemistry* **1973**, *12*, 3537-3542.

(2) Chari, R. V. J.; Whitman, C. P.; Kozarich, J. W.; Ngai, K.-L.; Ornston, L. N., preceeding paper.

(3) The enol-lactone, (2*R*,5*R*)-2,5-di[²H]-4-carboxymethylbut-3-en-4-olide can be generated enzymatically and its fate followed by 400-MHz NMR analysis. After 1 h we observed complete loss of the signal corresponding to the proton at the 2 position.

(4) Ornston, L. N.; Stanier, R. Y. *J. Biol. Chem.* **1966**, *241*, 3776-3786.

(5) 4-Carboxymuconate cycloisomerases from *P. putida* and *A. calcoaceticus* were purified as described previously.[2] The isomerase from *P. putida* was isolated according to Meagher and Ornston (Meagher, R. B.; Ornston, L. N. *Biochemistry* **1973**, *12*, 3523-3530). Purification of the isomerase from *A. calcoaceticus* followed the procedure of Patel et al. (Patel; R. N.; Meagher, R. B.; Ornston, L. N. *J. Biol. Chem.* **1974**, *249*, 7410-7419). The decarboxylase from *P. putida* was obtained by published procedures.[9] The *A. calcoaceticus* decarboxylase was isolated simultaneously with the cycloisomerase.[2] All enzymes were exchanged into ^2H_2O by use of a Centricon-10™ microconcentrator and equilibrated in ^2H_2O for 24 h before the experiment. Buffer (250 mM $K^2H_2PO_4$; pH 5.8, pD 6.2) and substrate (40 nm) were exchanged in ^2H_2O 3 times. All NMR spectra were recorded on a Brüker AM 400-MHz spectrometer. Chemical shifts are standardized to an $^1H^2HO$ resonance of 4.7 ppm. Enzymes were >95% homogeneous as determined by SDS gel electrophoresis.

(6) **1**: ¹H NMR (^2H_2O) δ 6.51 (d, 1 H, J = 12 Hz), 6.25 (s, 1 H), 5.78 (d, 1 H, J = 12 Hz).

(7) **2**: (4*R*,5*R*)-5-[²H]-4-carboxymuconolactone: ¹H NMR (^2H_2O) δ 7.54 (d, 1 H, J = 5.7 Hz), 5.96 (d, 1 H, J = 5.9 Hz), 2.89 (br s, 1 H). **3**: (2*R*,5*R*)-2,5-di[²H]-4-carboxymethylbut-3-en-4-olide: ¹H NMR (^2H_2O) δ 5.26 (s, 1 H), 3.14 (s, 1 H), 3.07 (br s, 1). The assignments for **3** were established by its quantitative formation from **2** by the decarboxylase.[3]

(8) **4**: (4*S*,5*R*)-2,5-[²H]-muconolactone: ¹H NMR (^2H_2O) δ 7.61 (s, 1 H), 5.32 (d, 1 H, J = 8.6 Hz), 2.30 (d, 1 H, J = 8.5 Hz). The lack of a significant resonance for the α-proton (6.02 ppm) establishes a nearly exclusive proton transfer from **3** to **4**. The final spectrum also corroborates the stereochemistry of C-5 for **2** as R^2 since the identical asymmetric collapse of the C-5 AB quartet is observed for the reaction of *cis,cis*-muconate with muconate cycloisomerase which yields (4*S*,5*R*)-5-[²H]muconolactone.

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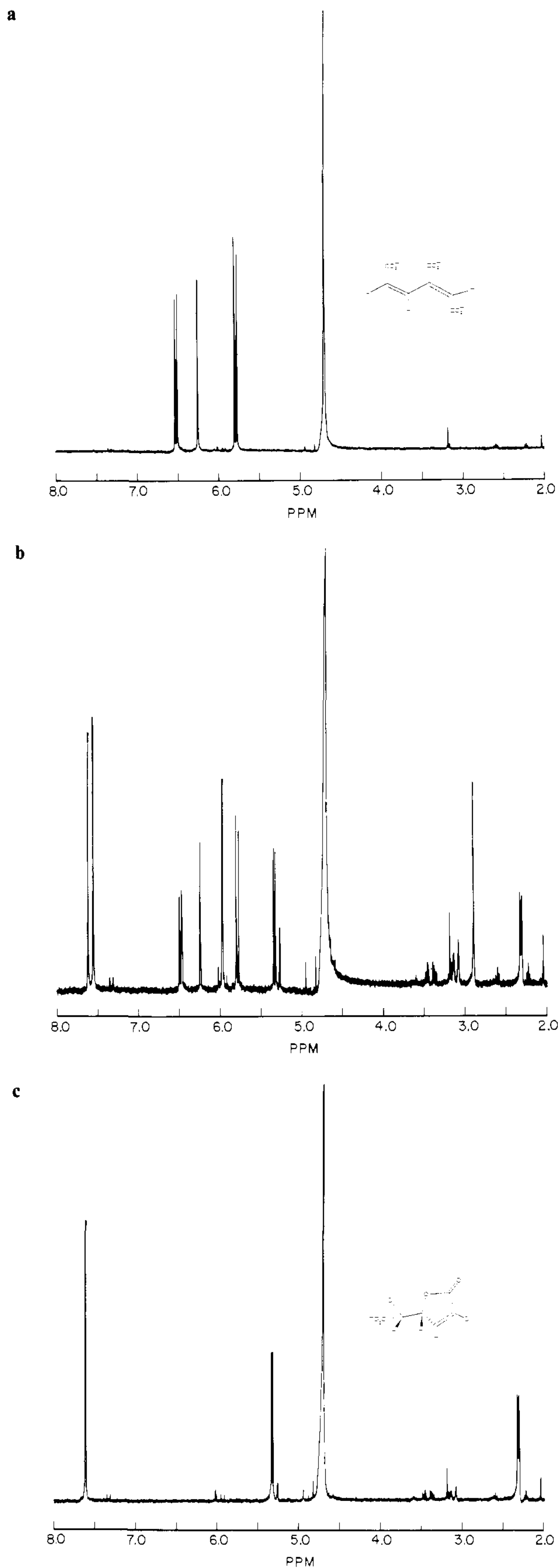
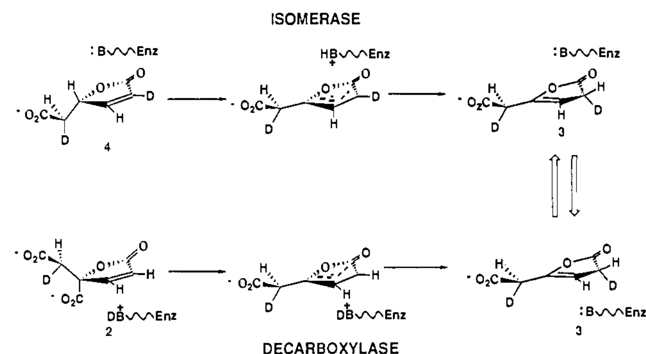


Figure 1. 400-MHz ^1H NMR (^2H_2O) spectra indicating the time course of the "ricochet" experiment for the *P. putida* enzymes: (a) 3-carboxy-*cis,cis*-muconate (**1**) in the presence of isomerase and decarboxylase; (b) 5 min after addition of 3-carboxymuconate cycloisomerase; (c) 15 min after addition of cycloisomerase. Identical results were obtained for the *A. calcoaceticus* enzymes.

Scheme II



enzyme which was capable of catalyzing both reactions.[9] Our stereochemical analysis suggests an intriguing possiblity to account for this enzymatic divergence. The ancestral enzyme would necessarily have the ability to perform chemistry on either face of a common (carbanionic) intermediate (Scheme II). The divergence of specific enzymatic activities could, therefore, have been due to the development of opposing facial recognition by both enzymes from their progenitor. Recent structural studies on the isomerase[10] and future work on the decarboxylase should be illuminating.

Note Added in Proof. We have recently completed stereochemical analyses of both the isomerase and decarboxylase by independent procedures by using appropriately labeled muconate derivatives. The preliminary results fully corroborate the conclusions of the "ricochet" analysis and will be reported elsewhere.

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(10) Katz, B. A.; Ollis, D.; Wyckoff, H. W. *J. Mol. Biol.* **1985**, *184*, 311–318.

ESR Investigation of the Cubane Radical Cation ($C_8H_8^+$) in Neon Matrices at 4 K

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The high strain energy (166 kcal/mol)[1] of the highly symmetric cubane molecule[2] and the unique bonding adopted by this stable hydrocarbon continues to provide extensive opportunity for fundamental investigation. Detailed analyses of its vibrational[3,4] and photoelectron spectra[5] have recently appeared. Also achieved have

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