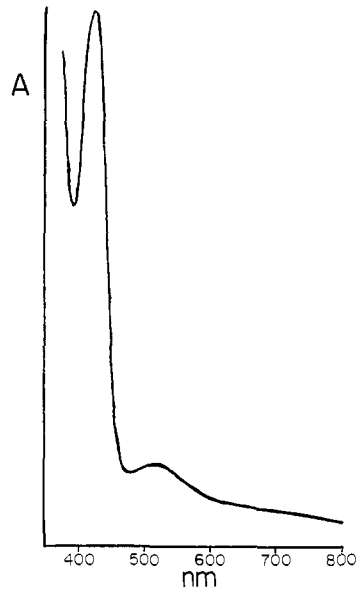


Figure 7. UV-vis spectrum of a dilute solution of $Na_2[Nb_2Cl_6(\mu\text{-}THT)_3]\cdot n$THF (**1b**) in THF.

energies. For $Nb_2Br_6(\mu\text{-}THT)_3^{2-}$ only one peak (at 19 300 cm^{-1}) was seen. The higher energy absorption seen in the chloride complexes was probably obscured in this case by a charge-transfer band which dominates the spectra at energies above 23 000 cm^{-1}.

In closing, we note a relevant recent development in this area. The compound $NaNb_3O_5F$ contains pairs of Nb(II) atoms at a distance of 2.61 Å from one another,[24] and this short distance led to the proposal of a triple Nb–Nb bond. The remainder of the coordination about each of the metal atoms is provided by a planar arrangement of four X atoms (X = O or F; these elements could not be distinguished crystallographically). The X atoms are eclipsed, and the Nb_2X_8 substructure is reminiscent of the class of structures typified by $Re_2Cl_8^{2-}$.[8] The metal–metal bonding picture is complicated by the presence of four Nb(IV) atoms at just over 3 Å away from the Nb(II) atoms. The extent to which these Nb(IV) atoms perturb the Nb(II)–Nb(II) triple bond and the exact nature of the bonding in this compound are uncertain. The experimental difficulties posed by this compound are such that these uncertainties will perhaps be resolved only through theoretical calculations.

Acknowledgment. We are grateful to the National Science Foundation and the Robert A. Welch Foundation for support of this work. M.P.D thanks the National Science Foundation for a NSF Predoctoral Fellowship and the Industry-University Cooperative Chemistry Program of Texas A&M for additional support.

Supplementary Material Available: Tables of anisotropic displacement parameters and complete listings of bond distances and bond angles for the crystal structures of **1c**, **3**, **4**, **5a**, and **5b** (22 pages); tables of observed and calculated structure factors for all five structures (85 pages). Ordering information is given on any current masthead page.

(24) Köhler, J.; Simon, A. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 996.

Absolute Stereochemical Course of the 3-Carboxymuconate Cycloisomerases from *Pseudomonas putida* and *Acinetobacter calcoaceticus*: Analysis and Implications[1]

Ravi V. J. Chari,[†] Christian P. Whitman,[‡] John W. Kozarich,*[§] Ka-Leung Ngai,[⊥] and L. Nicholas Ornston[⊥]

Contribution from the Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742, Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510, and Department of Biology, Yale University, New Haven, Connecticut 06511. *Received February 23, 1987*

Abstract: The absolute stereochemical course of the 3-carboxymuconate cycloisomerases [EC 5.5.1.2; 2-carboxy-5-oxo-2,5-dihydrofuran-2-acetate lyase (decyclizing)] from *Pseudomonas putida* and *Acinetobacter calcoaceticus* has been determined by chemical and ^1H NMR methods. The product of the enzyme-catalyzed reaction in ^2H_2O was detected by NMR and trapped by catalytic hydrogenation to afford 5-[^{2}H]homocitrate lactone. Subsequent chemical degradation of the monodeuteriated homocitrate lactone gave (2*R*,3*S*)-2-[^{2}H]citrate as determined by ^1H NMR analysis. The product of the cycloisomerase reaction was established as (4*R*,5*R*)-5-[^{2}H]-4-carboxymuconate, indicating that the lactonization proceeded by an anti addition—the mechanistic and stereochemical antipode of the previously studied muconate cycloisomerase from *P. putida* and 3-carboxymuconate cycloisomerase from *Neurospora crassa*. The anti addition probably represents the lower energy pathway for the reaction and suggests that the evolutionary relationship between the two classes of cycloisomerases is more remote than previously believed.

The *3-cis,cis*-carboxymuconate cycloisomerases [EC 5.5.1.2; 2-carboxy-5-oxo-2,5-dihydrofuran-2-acetate lyase (decyclizing)] from *Pseudomonas putida* and *Acinetobacter calcoaceticus* catalyze the lactonization of 3-caraboxy-*cis,cis*-muconate (**1**) to yield 4-carboxymuconolactone (**2**).[2] This unusual reaction is a key step in the catabolism of protocatechuic acid, which, along with the battery of enzymes responsible for catechol catabolism, comprises the β-ketoadipate pathway (Scheme I).

[†] Yale–Celanese Fellow. University of Maryland and Yale University School of Medicine.

[‡] NIH Postdoctoral Fellow. University of Maryland.

[§] American Cancer Society Faculty Research Awardee. University of Maryland and Yale University School of Medicine. Address correspondence to this author at the University of Maryland.

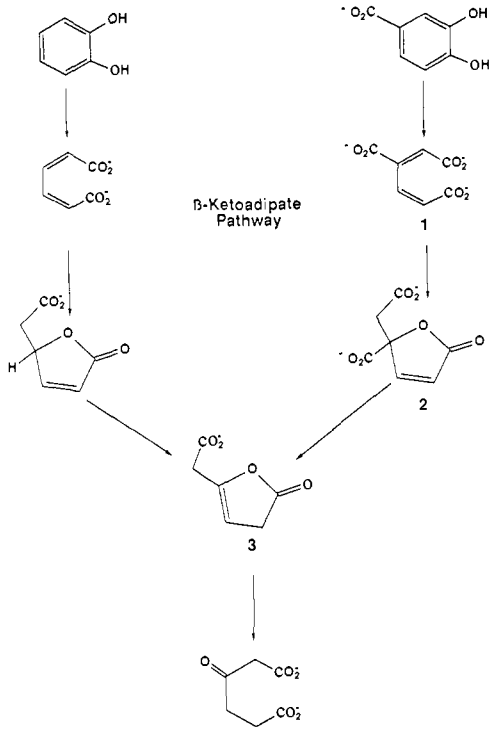
[⊥] Department of Biology, Yale University.

(1) For a preliminary account of this work, see: Kozarich, J. W.; Chari, R. V. J.; Ngai, K.-L.; Ornston, L. N. In *Mechanisms of Enzymatic Reactions: Stereochemistry*; Frey, P. A., Ed.; Elsevier: New York, 1986; pp 233–246.

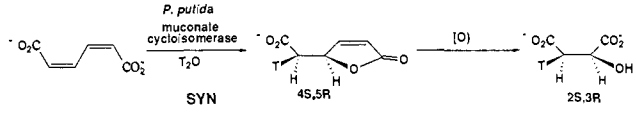
(2) (a) Ornston, L. N.; Stanier, R. Y. *J. Biol. Chem.* **1966**, *241*, 3776–3786. (b) Patel, R. N.; Meagher, R. B.; Ornston, L. N. *Biochemistry* **1973**, *12*, 3531–3537.

0002-7863/87/1509-5514$01.50/0

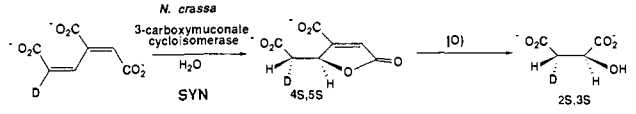
Scheme I



Scheme II



Scheme III



The striking similarity of analogous reactions found in the two converging branches of the pathway has been an obvious factor in its acceptance as a paradigm for the metabolic diversification of an ancestral enzyme.[3] The incomplete stereochemical record of these enzymes has supported this view. In 1969 Avigad and Englard[4] determined the absolute stereochemical course of muconate cycloisomerase from *P. putida* [EC 5.5.1.1; 5-oxo-2,5-dihydrofuran-2-acetate lyase (decyclizing)] (Scheme II). The reaction was performed in ^3H_2O, and the resulting 5-[^{3}H]-muconolactone was chemically oxidized to 3-[^{3}H]malate, which was determined to be the 2*S*,3*R* isomer by fumarase analysis. This established the cycloisomerase product as (4*S*,5*R*)-5-[^{3}H]-muconolactone and the mechanism of its formation as a syn process. In 1975 Kirby et al.[5] performed a similar stereochemical analysis on the 3-carboxymuconate cycloisomerase [EC 5.5.1.5; 3-carboxy-5-oxo-2,5-dihydrofuran-2-acetate lyase (decyclizing)] from *Neurospora crassa*, a eukaryote (Scheme III). This enzyme, in contrast to the prokaryotic title enzymes, catalyzes the opposite regiocyclization to yield 3-carboxymuconolactone. The analysis, a variation of the Avigad and Englard procedure,[4] established the reaction as a syn addition with the same stereochemical course as the muconate cycloisomerase. The conservation of mechanism and stereochemistry in the two cycloisomerases has been a keystone for the support of a common ancestral enzyme and has prompted the suggestion that the cyclization may involve "unique steric constraints" which favor a syn mechanism over an anti process.[6]

The determination of the absolute stereochemistry of **2** presents an interesting challenge since **2** undergoes spontaneous chemical decarboxylation to afford the enol–lactone **3** ($t_{1/2} \simeq 5$ min at pH 7.0), thereby destroying the chirality at C-4. Moreover, the characterization of **2**[1] has rested on its spontaneous decarboxylation to **3** and the fact that its chemical properties are distinct from those of 3-carboxymuconolactone, the product of the 3-carboxymuconate cycloisomerase from *Neurospora crassa*.[5] We wish to report the direct observation of stereospecifically monodeuteriated 4-carboxymuconolactone (**2**) by ^{1}H NMR and the elucidation of the absolute stereochemical course of its formation by the 3-carboxymuconate cycloisomerases from *P. putida* and from *A. calcoaceticus*. This has been accomplished by reductive trapping of **2** in situ by catalytic hydrogenation and by the stereochemical analysis of the resulting 5-[^{2}H]homocitrate lactone. The results suggest that profound mechanistic and stereochemical differences exist between the prokaryotic 3-carboxymuconate cycloisomerases and the two enzymes previously studied. A hypothesis is offered to account for these differences.

Results

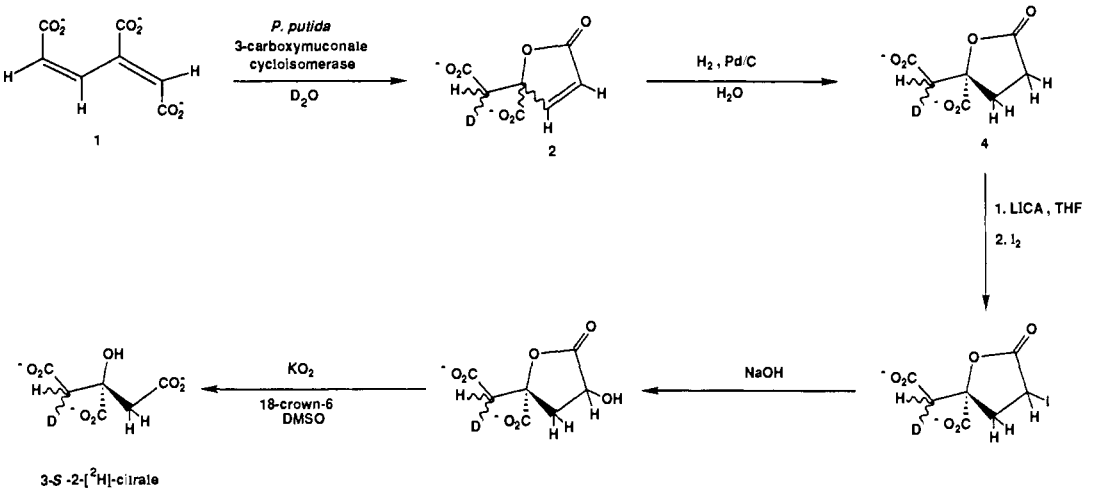
^{1}H NMR Detection of 4-Carboxymuconolactone. The rapid ^{1}H NMR analysis of the lactonization of 3-carboxymuconate in ^2H_2O (99%) by the cycloisomerase from *P. putida* is shown in Figure 1. The key feature of the spectrum is the broadened singlet at δ 2.95 ppm, which we attribute to the proton of the stereospecifically monodeuteriated carboxymethyl group. This assignment is corroborated by the spectrum of an identical reaction performed in the presence of 4% ^1H_2O (Figure 1, inset). Under these conditions this singlet is clearly superimposed on the vestiges of an AB quartet (J = 15 Hz) for the diprotiocarboxymethyl group. The asymmetric collapse of this quartet is indicative of a stereospecific deuteriation by the enzyme. Overall, the spectrum is clearly consistent with the structure of **2**. Upon prolonged standing (>1 h) at room temperature, **2** was found to undergo a nonenzymatic decomposition to afford a ^{1}H NMR spectrum consistent with the formation of the enol–lactone **3**.[7] This finding is in agreement with the reported spontaneous decarboxylation of **2**.[2] An analogous reaction performed with the *A. calcoaceticus* enzyme afforded identical results, indicating the same stereochemical course for this reaction.

Reductive Trapping of 4-Carboxymuconolactone. Since the chemical instability of **2** is primarily due to the conjugation of the carboxyl group to the α,β-unsaturated lactone, reduction of the α,β-double bond would prevent decarboxylation and presumably lead to a single stereoisomer of homocitrate lactone (**4**) (Scheme IV). Immediate catalytic reduction of **2** generated by the *P. putida* cycloisomerase in ^1H_2O using 20% Pd/C and H_2 afforded **4** in 85% overall yield from **1**. The ^{1}H NMR spectrum (Figure 2A) was identical with that of an authentic sample of racemic **4**. A similar reduction of **2** prepared in ^2H_2O yielded monodeuteriated **4**, the ^{1}H NMR spectrum of which revealed the characteristic asymmetric collapse of the AB quartet by loss of the upfield resonance (Figure 2B).

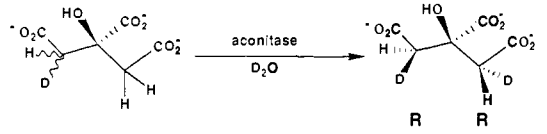
The 3*S* isomer of homocitric acid lactone has been unambiguously synthesized from (−)-quinic acid.[8] The optical rotation was found to be $[\alpha]^{21}_{589}$ +60.0° (c 0.55, water). We have determined the optical rotation of homocitric acid lactone isolated from four independent reductive trapping experiments performed in ^1H_2O; the values range from +54 to 58° under conditions essentially identical with those previously reported.[8] The monodeuteriated **4** afforded an optical rotation of +42°, reflecting an

(3) Clarke, P. H. In *Microbial Degradation of Organic Compounds*; Gibson, D. T., Ed.; Marcel Dekker: New York, 1984; Vol. 13, pp 11–27.
(4) Avigad, G.; Englard, S. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1969**, *28*, 345, Abstr. 486. Since this result has never been reported in a primary publication, we have verified the stereochemical course of this enzyme by a modification of their procedure.
(5) Kirby, G. W.; O'Laughlin, G. J.; Robins, D. J. *J. Chem. Soc., Chem. Commun.* **1975**, 402–403.
(6) Hanson, K. R.; Rose, I. A. *Acc. Chem. Res.* **1975**, *8*, 1–10.
(7) Chari, R. V. J.; Whitman, C. P.; Kozarich, J. W.; Ngai, K.-L.; Ornston, L. N. *J. Am. Chem. Soc.*, accompanying paper in this issue.
(8) Thomas, U.; Kalyanpur, M. G.; Stevens, C. M. *Biochemistry* **1966**, *5*, 2513–2516.

Scheme IV



Scheme V

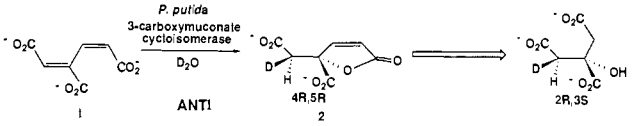


expected change in the magnitude of the rotation upon monodeuteration. We conclude that (3*S*)-homocitric acid lactone is produced in the reduction of 4-carboxymuconolactone (**2**) and, therefore, assign the stereochemistry of C-4 of **2** as *R* (priority numbering changes upon unsaturation of C-3).

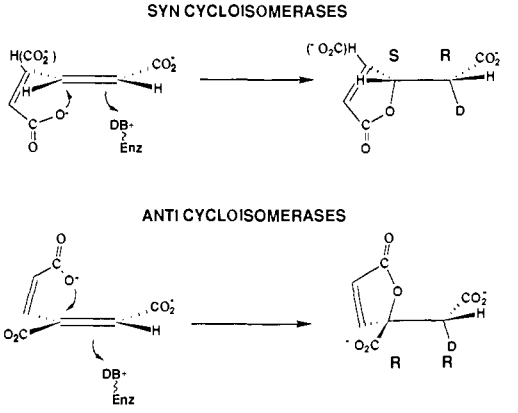
Conversion of (3*S*)-2-[^{2}H]Homocitric Acid Lactone to (3*S*)-2-[^{2}H]Citrate. Since the NMR resonances of the prochiral hydrogens of **4** have not been unambiguously assigned, we developed a procedure (Scheme IV) for the chemical conversion of monodeuteriated (3*S*)-**4** to the corresponding monodeuteriated (3*S*)-citrate, whose absolute stereochemistry could be determined. Thus, advantage was taken of the acidic α-protons of the lactone ring to generate the carbanion with lithium isopropylcyclohexylamide in THF (−78 °C) followed by iodination.[9] The α-iodo lactone was not isolated but was treated with mild aqueous sodium hydroxide to yield the α-hydroxyl lactone and the ring-opened acid. This mixture was oxidatively cleaved with potassium superoxide in the presence of 18-crown-6 in DMSO[10] to afford citrate, which was purified by ion-exchange chromatography.

^{1}H NMR Analysis of (3*S*)-2-[^{2}H]Citrate. The ^{1}H NMR spectrum of protio citrate in ^2H_2O is a simple one consisting of two superimposed AB quartets ($\Delta\delta \sim 0.12$ ppm; $J_{AB} = 15$ Hz) for the two magnetically nonequivalent protons of the two equivalent methylene groups.[11] The resonances have been assigned by reaction of citrate in ^2H_2O with aconitase.[12] Aconitase [EC 4.2.1.3; citrate (isocitrate) hydrolase] is known to exchange the *pro-R* proton of the *pro-R* arm of citrate.[13] The ^{1}H NMR spectrum of the resulting (2*R*,3*R*)-2-[^{2}H]citrate has been shown to be a broadened upfield singlet superimposed upon an AB quartet due to the protons of the *pro-S* arm.[12] The downfield doublet of citrate is thus assigned to H_R of the *pro-R* arm and H_S of the *pro-S* arm; H_S of the *pro-R* arm and H_R of the *pro-S* arm must, therefore, yield the upfield doublet. An identical spectrum has been reported for (2*S*,3*S*)-2-[^{2}H]citrate prepared by stereoselective deuteriation of a synthetic precursor.[14]

Scheme VI



Scheme VII



The ^{1}H NMR spectrum of the monodeuteriated citrate derived from the cycloisomerase reaction is shown in Figure 3A. Loss of an upfield proton relative to citrate is immediately apparent, with the corresponding downfield broadened singlet partially obscuring the remaining AB quartet. Since the optical rotation of the precursor homocitric acid lactone establishes the stereochemistry of C-3 as *S*, the deuteriated position must be H_R of the *pro-S* arm. Treatment of this monodeuteriated citrate with aconitase in ^2H_2O corroborates the analysis (Scheme V). The remaining two protons of the resulting dideuteriated citrate are predicted to be nonequivalent, thereby affording a spectrum of two broadened singlets. Figure 3B shows this nonequivalence of proton resonances and establishes the monodeuteriated citrate as (2*R*,3*S*)-2-[^{2}H]citrate.

Absolute Stereochemistry of 3-Carboxymuconate Cycloisomerase. By the chemistry discussed above, the (2*R*,3*S*)-2-[^{2}H]citrate must be uniquely derived from (2*R*,3*S*)-2-[^{2}H]-homocitric acid lactone, which in turn must be the product of the catalytic hydrogenation of (4*R*,5*R*)-5-[^{2}H]-4-carboxymuconolactone (Scheme VI). This product can only be formed from 3-carboxymuconate by an anti addition. The same conclusion is reached for the *A. calcoaceticus* cycloisomerase since the ^{1}H NMR

(9) Rathke, M. W.; Lindert, A. *Tetrahedron Lett.* **1971**, 3995–3998.
(10) San Filippo, J., Jr.; Chern, C.-I.; Valentine, J. S. *J. Org. Chem.* **1976**, *41*, 1077–1078.
(11) Bovey, F. *Nuclear Magnetic Resonance Spectroscopy*; Academic Press: New York, 1969; p 98.
(12) Villafranca, J. J.; Mildvan, A. S. *J. Biol. Chem.* **1972**, *247*, 3454–3463.
(13) Hanson, K. R.; Rose, I. A. *Proc. Natl. Acad. Sci. U.S.A.* **1963**, *50*, 981–984.
(14) Brandänge, S.; Dahlman, O. *J. Chem. Soc., Chem. Commun.* **1983**, *1324*–1325.

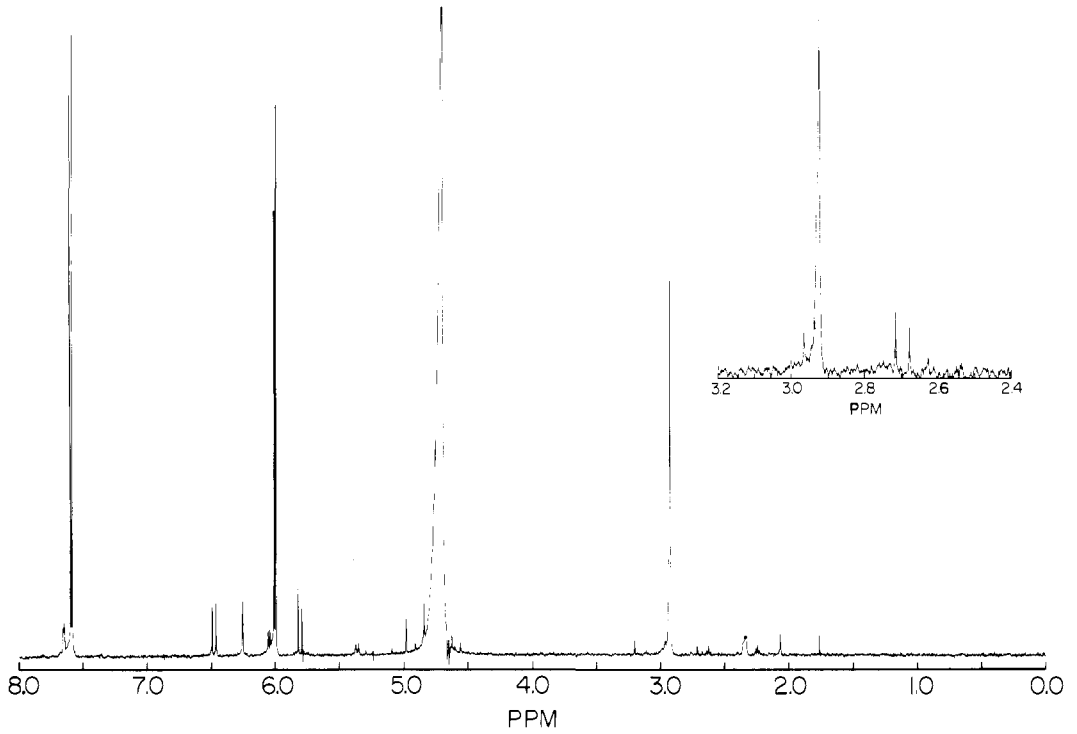


Figure 1. ^1H NMR (400 MHz) spectrum of 4-carboxymuconolactone (**2**) generated by cycloisomerase-catalyzed lactonization of 3-carboxy-*cis*,-*cis*-muconate (**1**) in ^2H_2O. An expanded spectrum of vestiges of an AB quartet for the diprotiocarboxymethyl group generated by identical cycloisomerase-catalyzed reaction in 4% ^1H_2O is also shown (inset).

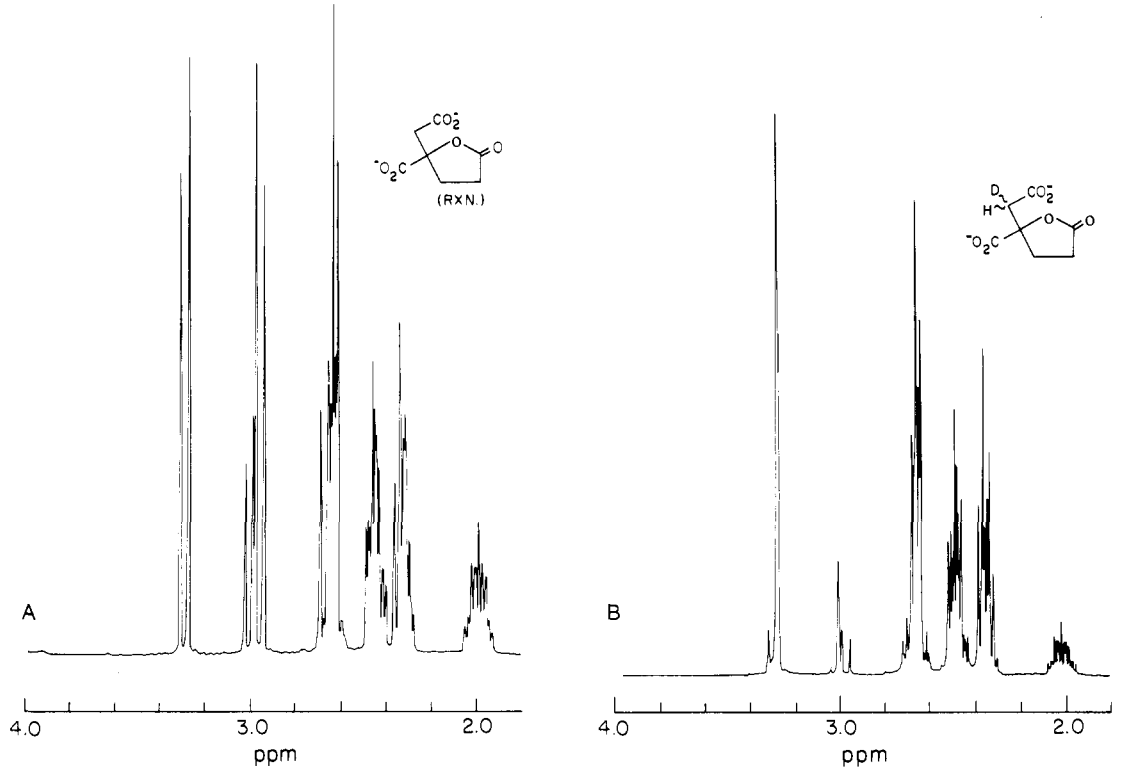


Figure 2. ^1H NMR (400 MHz) spectra of (A) (*S*)-homocitrate lactone (**4**) generated by catalytic hydrogenation of 4-carboxymuconolactone (**2**), which is the product of cycloisomerase-catalyzed lactonization of 3-carboxy-*cis*,*cis*-muconate (**1**) in ^1H_2O, and (B) monodeuteriated (*S*)-homocitrate lactone (**4**) generated in an identical reaction by the cycloisomerase reaction performed in ^2H_2O.

spectrum of the monodeuteriated **2** obtained for the reaction of this enzyme with **1** in ^2H_2O is identical with that found for the *P. putida* enzyme (Figure 1).

Discussion

Our results clearly indicate that the title enzymes proceed by a profoundly different stereochemical and mechanistic course than the previously studied muconate cycloisomerase from *P. putida*[4] and 3-carboxymuconate cycloisomerase from *N. crassa*.[5] A cataloging of the four enzymatic reactions by syn or anti mechanism (Scheme VII) reveals the formation of opposing relative configurations at the carbon that has undergone attack by the incoming carboxylate moiety. This would appear to require that the syn and anti enzymes recognize different mirror-image, out-of-plane conformers of the muconate substrate. This observation by itself suggests that the evolutionary relationship of these enzymes is

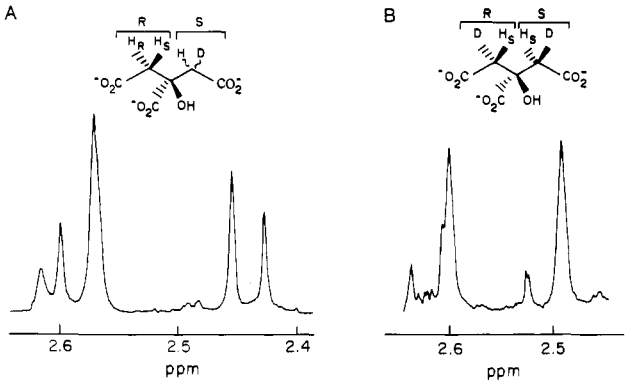


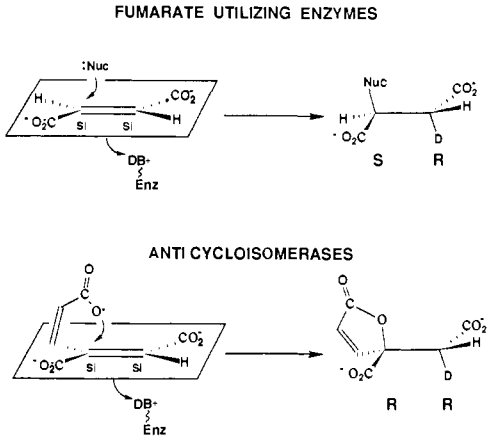
Figure 3. ^1H NMR (400 MHz) spectra of (A) monodeuteriated citrate obtained by chemical conversion of (*S*)-homocitrate lactone and (B) dideuteriated citrate formed by aconitase-catalyzed exchange of monodeuteriated citrate in ^2H_2O.

considerably more remote than previously believed.

The argument for a common ancestral cycloisomerase is founded on the tacit assumption that the active-site scaffolding required for catalysis (and, therefore, binding of the transition state) remains more or less immutable. The acquisition of substrate specificity can be achieved by mutations that result in the accommodation or enhanced binding of substrate functional groups peripheral to the structure and energetics of the transition state, a variation of the lock-and-key hypothesis. The carboxyl group of 3-carboxymuconate has fallen victim to this idea, and its relegation to a minor role in substrate recognition has been reinforced by the previous stereochemical studies.[4,5] The observation of identical mechanisms for the prokaryotic muconate and eukaryotic 3-carboxymuconate cycloisomerases argues strongly for a close relationship between these syn enzymes. Indeed, in the cyclization of 3-carboxymuconate to 3-carboxymuconolactone by the *N. crassa* cycloisomerase, the 3-carboxyl group would not appear to have a significant impact on the addition to the opposing double bond vis-à-vis the muconate cyclization (Scheme VII, top). In contrast, the cyclization of 3-carboxymuconate to 4-carboxymuconolactone by the title enzymes might be anticipated to be substantially altered in mechanism by this carboxyl group (Scheme VII, bottom). Its direct effects on the electronic character of the electrophilic double bond and on the transition state of the reaction could necessitate an entirely different active-site structure and evolutionary history for these enzymes. It this is the case, then what is the lineage of the "anti" cycloisomerases?

We would like to offer a possibility. A simple distinction between the syn and anti cycloisomerases may be made based on the reacting double bond. The syn enzymes catalyze an intramolecular 1,2-addition on a double bond bearing a single carboxyl group; the anti enzymes catalyze a similar addition on a double bond bearing two carboxyl groups in a trans relationship. Hanson and Rose[6] have noted that enzyme-catalyzed 1,2-eliminations (1,2-additions) which form a trans or cis double bond between two carboxylate groups all occur by anti mechanisms. Notable examples are fumarase, aspartase, argininosuccinate lyase, adenylosuccinate adenylate lyase, and aconitase. It is quite likely that some of these share a common ancestor.[15] A comparison of the anti cycloisomerases to these enzymes is compelling. The fumarate-dependent enzymes (Scheme VIII, top) exhibit, in addition to an anti mechanism, the same overall absolute stereochemical course regardless of the identity of the nucleophile. The prokaryotic 3-carboxymuconate cycloisomerases proceed with the same stereochemistry as the fumarate enzymes and may be

Scheme VIII



considered to catalyze an intramolecular addition to a fumarate moiety (Scheme VIII, bottom). It is tempting to speculate, then, that the anti cycloisomerases arose by divergent evolution from a fumarate-dependent ancestor with conservation of active-site structure.[6]

The syn and anti cycloisomerases may reflect a fundamental natural selection for catalytic efficiency between the minimal number of catalytic groups and the maximum separation of those groups or of those in the substrate. Syn mechanisms are generally consistent with economy in the use of catalytic groups since the chemistry is restricted to one face of the substrate.[6] For the syn cycloisomerases, the price for simplicity might well be the development of eclipsing (to a first approximation) interactions within the transition state and product, assuming minimal substrate motion during catalysis.[6] Clearly, the energetic cost was not prohibitively high and the minimal number rule took precedence. A syn cyclization of 3-carboxymuconate to 4-carboxymuconolactone, however, would result in an additional carboxyl–hydrogen eclipsing interaction and a less-than-optimum separation of the two carboxyl groups in the product. These factors, in addition to the decreased polarity of the double bond, appear to have raised the energy of the transition state of the syn mechanism to a level where the minimal number rule was sacrificed for maximum separation of catalytic and substrate functional groups. The resulting anti mechanism leads to staggered interactions and maximal carboxylate separation, alleviating some of the energetic burden of the syn transition state.

Little additional evidence in support of this hypothesis is presently available. The syn and anti cycloisomerases differ markedly in their dependence of metal ions for catalysis. Muconate cycloisomerase has a requirement for a divalent metal ion with the highest specificity for manganese, which appears to be located in the active site.[16] An X-ray structure for this enzyme has been reported.[17] The most striking feature of the molecule is the occurrence of an α,β-barrel[17b]—a structure closely associated with a number of enzymes that stabilize carbanion intermediates. The amino acid sequence has been established from the sequence of the gene.[18] The anti cycloisomerases do not appear to have a metal requirement,[2] an observation in agreement with the properties of the fumarate-dependent enzymes. The entire amino acid sequence has yet to be determined, although N-terminal analysis suggests at least some homology with the muconate cycloisomerases.[2]

The elucidation of the underlying principles behind the evolution of enzyme specificities has been an area of increased activity in recent years.[19] A recent debate has focused on the facial spe-

(15) A strong structural and functional homology has been reported for fumarase and apartase: Woods, S. A.; Miles, J. S.; Roberts, R. E.; Guest, J. R. *Biochem. J.* **1986**, *237*, 547–557. The inclusion of aconitase is not entirely appropriate to the discussion of fumarate-dependent enzymes. The active-site structure of aconitase is clearly more complicated as is the overall conversion of citrate to isocitrate which it catalyzes: Robbins, A. H.; Stout, C. D. *J. Biol. Chem.* **1985**, *260*, 2328–2333; Emptage, M. H.; Dreyer, J.-L.; Kennedy, M. C.; Beinert, H. *Ibid.* **1983**, *258*, 11106–11111.

(16) (a) Ngai, K.-L.; Ornston, L. N.; Kallen, R. G. *Biochemistry* **1983**, *22*, 5223–5230. (b) Ngai, K.-L.; Kallen, R. G. *Ibid.* **1983**, *22*, 5231–5236.

(17) (a) Goldman, A.; Ollis, D. L.; Ngai, K.-L.; Steitz, T. A. *J. Mol. Biol.* **1985**, *182*, 353–355. (b) Goldman, A.; Ollis, D. L.; Steitz, T. A. *Ibid.* **1987**, *194*, 143–153.

(18) Aldrich, T. L.; Frantz, B.; Gill, J. F.; Chakarbarty, A. M., submitted for publication.

cificity for nicotinamide adenine dinucleotide (NAD) by a number of dehydrogenases.[20] The "stereochemical imperative" is believed to be a reflection of energetics; syn and anti forms of NAD differ in redox potential and may be required to match the potential of the substrate carbonyl. Concern over this hypothesis has been expressed since the physiological functions of and substrates for some of the included dehydrogenases may not be well established. This aside, the number of tests that a dehydrogenase must pass in order to be included in the hypothesis suggests that the NAD-dependent dehydrogenases are a too inherently complex class of enzymes to serve as a paradigm. The simplicity of the cycloisomerase model, then, is particularly significant. There can be no question of the highly specialized function of these enzymes or of their physiological substrates. The mechanistic and stereochemical differences reported here offer a straightforward lesson in the importance of chemistry and energetics in the evolution of enzymes that recognize nearly identical substrates. Additional studies to probe the relationship of the cycloisomerases are in progress.

Experimental Section

Materials. All solvents and chemicals were obtained from Aldrich Chemical Co., with the exception of 3-carboxy-*cis*,*cis*-muconate, which was prepared according to the procedure of Ornston and Stanier.[2a] Hydrogen and nitrogen were from Air Products Inc. All biochemicals, buffers, and resins were purchased from Sigma Chemical Co. Centricon (10 000 MW cutoff) centrifugal microconcentrators were obtained from Amicon. Components for LB broth were obtained from American Scientific Products.

Methods. Protein concentrations were determined colorimetrically by the Bradford method.[21] HPLC was performed on a Waters system using a Toya Soda TSK DEAE-5PW anion-exchange column (10-μm particle diameter). NMR spectra were obtained on a Brucker WM 500 or a Brucker AM 400 NMR spectrometer (Yale University and University of Maryland, respectively). Chemical shifts were standardized to a $^1H^2HO$ resonance at 4.7 ppm.

Purification of 3-Carboxymuconate Cycloisomerase from *P. putida*. Cultures of *Pseudomonas putida* A.3.12 (American Type Culture Collection 12633) were grown on *p*-hydroxybenzoate at 30 °C. Purification of the cycloisomerase was carried out according to published procedures.[2b] The enzyme had a final specific activity of 800 units/mg. A unit of enzyme catalyzes the consumption of 1 μmol of 3-carboxy-*cis*,*cis*-muconate per minute at pH 8.0.

Purification of 3-Carboxymuconate Cycloisomerase from *A. calcoaceticus*. Cultures of *Escherichia coli* JM 109 containing recombinant plasmid pCAl carrying the genes for the enzymes of the protocatechuate pathway from *Acinetobacter calcoaceticus* were grown at 37 °C in LB broth containing ampicillin (100 μg/mL) and isopropyl-β-D-thiogalactosylpyranoside (IPTG; 1 mM).[22] Frozen cells (2.5 g) were suspended in 10 mL of 50 mM sodium ethylenediamine (pH 7.3) containing 1 mM 2-mercaptoethanol. The cells were disrupted at 0 °C with five pulses (30 s each) by a Brausonic 2000 sonicator equipped with the 8.5-mm 12T probe (50-W output). The solution was vortexed vigorously for a few minutes and centrifuged at 15000 *g* for 15 min. The supernatant was collected and dialyzed overnight against 1 L of 10 mM sodium ethylenediamine (pH 7.3) containing 1 mM 2-mercaptoethanol. The solution was centrifuged at 100000 g for 30 min to remove particulate matter. The supernatant was syringe filtered through a 0.22-μm Millex GV (low binding) unit. Ten milliliters of this extract (20–25 mg/mL protein) was injected into a TSK DEAE-5PW column (21.5 mm × 15 cm) equilibrated with ethylenediamine buffer with a flow rate of 5 mL/min. The column was washed with buffer for 10 min and elution was carried out with a 0–0.25 M sodium chloride gradient in 120 min. The eluant was monitored by UV detection (280 nm), and 10-mL fractions were collected on ice and assayed for cycloisomerase activity. Active fractions were pooled and concentrated to 100 μL in ethylenediamine buffer with an Amicon Centricon-10 ultrafilter (final specific activity = 500 units/mg).

^1H NMR Detection of 4-Carboxymuconolactone. To a NMR tube was added a 40 mM solution of 3-carboxy-*cis*,*cis*-muconate trisodium salt (6 mg/0.6 mL) containing 100 mM $K[^2H_2]PO_4$ (pD 7.4) in ^2H_2O. A final concentration of 4% ^1H_2O was achieved by addition of 25 μL of H_2O. Solutions of 3-carboxymuconate cycloisomerase were exchanged in ^2H_2O by repeated centrifugation of the enzyme in ^2H_2O in a Centricon-10 microconcentrator. The reaction was initiated by addition of enzyme (20 μL), and the ^1H NMR spectrum was taken after 30 min. ^1H NMR: for 5-[^{2}H]-3, δ 2.92 (1 H, br s, $CHDCO_2^-$), 5.96 (1 H, d, J = 5.8 Hz, Hα), 7.54 (1 H, d, J = 5.8 Hz, Hβ); for diprotio **3**, δ 2.69 (1 H, d, J = 15 Hz), 2.94 (1 H, d, J = 15 Hz).

Catalytic Reduction of 5-[^{2}H]-4-Carboxymuconolactone. After complete enzymatic lactonization, the reaction mixture consisting of 4-carboxymuconolactone (372 mg; 2 mmol) in ^2H_2O (40 mL) was diluted with ^1H_2O (460 mL) and immediately treated with 20% Pd/C (250 mg). Hydrogen gas was passed rapidly through the solution for 1.5 h. The catalyst was removed by filtration, and the resulting monodeuteriated homocitrate lactone was isolated by chromatography on a Dowex-1 (formate) column (1.5 × 20 cm), eluting with an aqueous formic acid gradient (0 → 4 M; 600 mL total volume). The product eluted at ~2.8 M formic acid as the major peak. Appropriate fractions were pooled and lyophilized (yield = 310 mg; 82%). ^1H NMR (^2H_2O): homocitrate lactone (protio), δ 2.28–2.39 (1 H, m), 2.41–2.49 (1 H, m), 2.61–2.67 (2 H, m), 2.92 (1 H, d, J = 20 Hz), 3.28 (1 H, d, J = 20 Hz); 5-[^{2}H]homocitrate lactone, δ 2.31–2.38 (1 H, m), 2.45–2.51 (1 H, m), 2.63–2.67 (2 H, m), 3.27 (1 H, br s).

Conversion of 5-[^{2}H]Homocitric Acid Lactone to 2-[^{2}H]Citrate. To a solution of isopropylcyclohexylamine (84 μL, 0.5 mmol) in anhydrous THF (4 mL) under N_2 at 0 °C was added *n*-butyllithium (0.5 mmol). The mixture was stirred for 1 h, followed by cooling to −78 °C. 5-[^{2}H]Homocitric acid lactone (20 mg, 0.1 mmol) in THF (1 mL) was added dropwise to the mixture, and the mixture was stirred for an additional 1 h at −78 °C. This solution was subsequently added (reverse addition) to a solution of I_2 (260 mg, 1 mmol) in THF (2 mL) at −78 °C and stirred for 20 min. The reaction was quenched with H_2O, and excess I_2 was removed by extraction with diethyl ether. The aqueous layer was adjusted to pH 9.0 with NaOH and then heated to 70 °C for 10 min. The solution was then passed through a small Dowex-H^+ column, and the eluant was evaporated and thoroughly dried in vacuo. The residue was taken up in dry DMSO (0.5 mL) and treated with potassium superoxide (56 mg, 0.8 mmol) and 18-crown-6 (52 mg, 2 mmol). The mixture was stirred at 30 °C for 24 h. Water (20 mL) was added and the crown ether was removed by ether extraction. The aqueous layer was subjected to chromatography on a Dowex-1 (formate) column (1 × 10 cm), eluting with an aqueous formic acid gradient (0 → 5 M, 600-mL total volume). Citric acid eluted at 2.5 M formic acid. Appropriate fractions were pooled and lyophilized. Overall yield of 2-[^{2}H]citric acid was determined to be 16% by enzymatic assay using citrate oxaloacetate lyase (EC 4.1.3.6) from *Enterobacter aerogenes* (Sigma; 0.25 units/mg) and L-malate dehydrogenase (EC 1.1.1.37; Sigma).[23]

^1H NMR Analysis of 2-[^{2}H]Citrate. The citrate sample (1 mg, 5 μmol) was dissolved in 0.1 M $K^2H_2PO_4$ in ^2H_2O (pD 7.0; 0.5 mL). Exchange of the *pro-R* proton of the *pro-R* arm of the citrate was accomplished by the addition of 25 μL of porcine heart aconitase (Sigma; 30 mg/mL) that had been activated according to published procedures.[24]

Acknowledgment. We thank the Yale–Celanese Program and the National Institutes of Health (Grant GM 37210 to J.W.K. and Grant GM 33377 to L.N.O.) for support of this research. The 400-MHz Bruker AM 400 spectrometer (University of Maryland) is supported by a grant from the National Science Foundation (Grant DMB-87-20175). We also thank Dr. Sudhir Ajmera for a helpful suggestion.

Registry No. 1, 109467-28-9; **2**, 13249-46-2; **2**-(*R*), 109467-29-0; 3-carboxymuconate cycloisomerase, 9075-77-8; homocitric acid lactone, 91912-46-8; citrate, 77-92-9; isopropylcyclohexylamine, 1195-42-2; *n*-butyllithium, 109-72-8.

(19) (a) Oppenheimer, N. J.; Marschner, T. M.; Malver, O.; Kam, B. L. In *Mechanisms of Enzymatic Reactions: Stereochemistry*; Frey, P. A., Ed.; Elsevier: New York, 1986; pp 15–28. (b) Benner, S. A.; Nambiar, K. P. *Ibid.*, pp 29–43.

(20) (a) Benner, S. A. *Experientia* **1982**, *38*, 633–637. (b) Nambiar, K. P.; Stauffer, D. M.; Kolodziej, P. A.; Benner, S. A. *J. Am. Chem. Soc.* **1983**, *105*, 5886–5890. (c) Oppenheimer, N. J. *Ibid.* **1984**, *106*, 3032–3034. (d) Benner, S. A.; Nambiar, K. P.; Chambers, G. K. *Ibid.* **1985**, *107*, 5513–5517.

(21) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.

(22) Doten, R. C.; Ngai, K.-L.; Mitchell, D. J.; Ornston, L. N., submitted for publication.

(23) Möllering, H.; Gruber, W. *Anal. Biochem.* **1966**, *17*, 369–376.

(24) Gawron, O.; Waheed, A.; Glaid, A. J.; Jaklitsch, A. *Biochem. J.* **1974**, *139*, 709–714.