isomer concentrations then evolve to achieve thermodynamic proportions following a phenomenological first-order process:

$$\text{reverse} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \text{normal}$$

with $k_f/k_r = K_{eq}$ and $k_f + k_r = k_{obsd}$. In wild-type metaquoMb, reorientation is slowest at neutral pH, and the observed rate $^{wt}k_{obsd}$ is ca. $3.7 \times 10^{-4}$ min$^{-1}$ at pH 7.5.[14] If this rate is maintained in His-82-Gln Mb, the mutant reverse isomer should be detectable for days. The reorientation is illustrated in Figure 1; a spectrum collected after 8 min of equilibration (Figure 1C) exhibits partially resolved reverse isomer peaks at the same positions as in wild-type Mb. Simulation and integration of spectrum 1B determine the equilibrium proportion of the two forms in His-82-Gln Mb. The equilibrium constant $^{82}K_{eq}$ is $\sim$20 compared to 11 in wild-type Mb. Thus, the His-82-Gln replacement destabilizes the minor form with respect to the major form by about 1.5 kJ mol$^{-1}$ at neutral pH.

When the area of peak m is followed as a function of reaction time (as in Figure 1C,D) and the data are fit to a first-order equation, $^{82}k_{obsd} \approx 2.2 \times 10^{-2}$ min$^{-1}$ is obtained; i.e., given $^{82}K_{eq}$, the rate constant $k_f$ is accelerated by a factor of $\sim$60. This implies a decrease in $\Delta G^{\circ}_f{}^{\ddagger}$ by $\sim$11 kJ mol$^{-1}$.[17] The value is larger than justified by the destabilization of the minor form, and the mutation must therefore affect the reverse barrier height ($\Delta G^{\circ}_r{}^{\ddagger}$) as well.[18]

Barrier lowering can arise from destabilization of the ground states, stabilization of the transition state(s), or both. Destabilization of the ground state was tested by denaturation experiments with the assumption that the unfolded state is negligibly affected by the mutation at pH 7.5. The urea denaturation of wild-type and His-82-Gln Mb is illustrated in Figure 2. The curve for His-82-Gln holoMb is significantly displaced to lower denaturant concentrations compared to wild-type holoMb. Although non-two-state behavior prevents quantitation of the effect, ground-state destabilization does account for at least some of the acceleration of heme reorientation. Figure 2 also contains the denaturation curves for the apoproteins; apoMb is only slightly affected, if at all, by the mutation.

The His-82-Gln Mb results show that the imidazole ring at that position has a stabilizing role in native holoMb but not in native apoMb. In addition, although residue 82 is not in contact with the heme, it has a marked effect on the kinetics of heme reorientation and influences the ability of the protein to favor one isomer over the other. Heme reorientation has been investigated extensively with modified hemins.[14,24] However, protein matrix alterations have not been explored to determine the mechanism, which is most certainly complex as it reflects the conformational and chemical changes required to relocate the large heme group. F-helix unfolding may facilitate heme release[16,25] and is consistent with the view that the helix is conformationally labile even in the holoprotein.[26] It is possible that the EF-H interface must also separate for the heme to turn around. Model building shows that a glutamine cannot assume the same geometry with respect to Asp-141 as a histidine and may weaken the interactions between the two elements of structure. Further speculation on the molecular origin of the effect will require that more sites be targeted. Our observations suggest that the dynamics and thermodynamics of a designed protein could be adjusted without modifying functionally important prosthetic group and binding site residues.

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## Characterization of a Dienol Intermediate in the 5-(Carboxymethyl)-2-oxo-3-hexene-1,6-dioate Decarboxylase Reaction

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5-(Carboxymethyl)-2-oxo-3-hexene-1,6-dioate decarboxylase (EC 4.1.1; COHED) from *Escherichia coli C* catalyzes the magnesium-dependent decarboxylation of **1** (Scheme I), a catabolite generated by the microbial degradation of (4-hydroxyphenyl)acetate.[1-3] The generally accepted mechanism for a $\beta$-decarboxylase that acts on an $\alpha$-keto acid involves the intermediate formation of a metal-stabilized enol which ketonizes to the $\alpha$-keto acid product.[4,5] In light of this mechanism, the COHED-catalyzed decarboxylation of **1** is particularly intriguing because the product is reportedly 2-hydroxy-2,4-heptadiene-1,7-dioate (**2**).[1,2,6] Because this conclusion rests solely on the observation and isolation of a compound with a $\lambda_{max}$ at 276 nm from a reaction mixture containing **1** and COHED and no further characterization of this compound has been reported,[1,2,6] we initiated a rigorous investigation of the COHED reaction. We find that COHED generates a mixture of **2** and the $\beta,\gamma$-enone, 2-oxo-4-heptene-1,7-dioate (**3**; Scheme II). Moreover, incubation of **2** with COHED produces **3**. These results suggest that COHED catalyzes the decarboxylation of **1** to **3** through the intermediacy of **2** (Scheme II). To our knowledge, this is the first report of the characterization of a dienol as an intermediate in a metal-dependent decarboxylase reaction.

The substrate for COHED, **1**, is generated by the action of 5-(carboxymethyl)-2-hydroxymuconate isomerase (CHMI) on **4**.[7]

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(7) The isolation of **4**, CHMI, and COHED have been described.[1-3,6] In our hands, the final specific activity of COHED ranges from 160 to 250 units/mg of protein.

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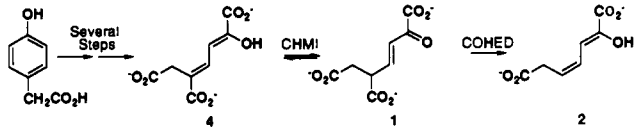
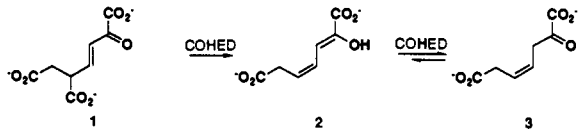
**Scheme I**




**Scheme II**




$^1H$ NMR analysis of the reaction shows a mixture of **1** and **4** (Figure 1A).[8] Addition of COHED (4 units) to the mixture produces a complex spectrum (Figure 1B) which verifies the presence of a set of signals corresponding to the previously reported product **2** and reveals the presence of a set of signals which is assigned to **3**.[9,10] Subsequent acidification of a similar mixture results in the isolation of a compound which is identified as **2**.[11]

These results immediately raise the question of whether the appearance of **3** is due to the nonenzymic ketonization of **2** or due to the ketonization of the enzyme-bound dienol.[12] This question was addressed by two experiments. First, the reactions of **2** with and without COHED are monitored by UV spectroscopy at 276 nm. Dienol **2** undergoes facile chemical decay in aqueous phosphate buffer (20 mM, pH 7.23).[13] Incubation of **2** with COHED ($2.8 \times 10^{-8}$ M) from *E. coli C* results in the same decay at 276 nm although at a faster rate. The observed rate is approximately 3-fold greater than the buffer-catalyzed rate.[14] A spectral scan showed that both reactions generate a mixture of **2** and a product without significant absorbance above 200 nm. The product was identified as (*Z*)-2-oxo-4-heptene-1,7-dioate (**3**) by $^1H$ NMR spectroscopy[9] and by its conversion to 2-hydroxy-4-heptene-1,7-dioate with $NaBH_4$.[15] Second, **1** and **2** were examined as substrates for COHED. Initial rates were measured for a range of concentrations of **1** (12–278 $\mu$M) in 20 mM $NaH_2PO_4$ buffer (pH 7.50, 30 °C) using an enzyme concentration of $2.8 \times 10^{-8}$ M by monitoring the decay of **1** at 236 nm ($\epsilon = 7.07 \times 10^3$ $M^{-1}$ $cm^{-1}$). Likewise, initial rates were measured for a range of concentrations of **2** (15–271 $\mu$M) under the same conditions by monitoring the decay of **2** at 276 nm ($\epsilon = 12.3 \times 10^4$ $M^{-1}$ $cm^{-1}$). Values of $K_M$, $k_{cat}$, and $k_{cat}/K_M$ were determined from Lineweaver–Burke plots and were found to be 65 $\mu$M, 54 $s^{-1}$, and $8.2 \times 10^5$ $M^{-1}$ $s^{-1}$ for **1** and 54 $\mu$M, 81 $s^{-1}$, and $1.5 \times 10^6$ $M^{-1}$ $s^{-1}$ for **2**. A comparison of the values for $k_{cat}/K_M$ and $k_{cat}$ indicates that **2** is an excellent substrate for COHED and that it is kinetically competent to be an intermediate in the overall reaction.

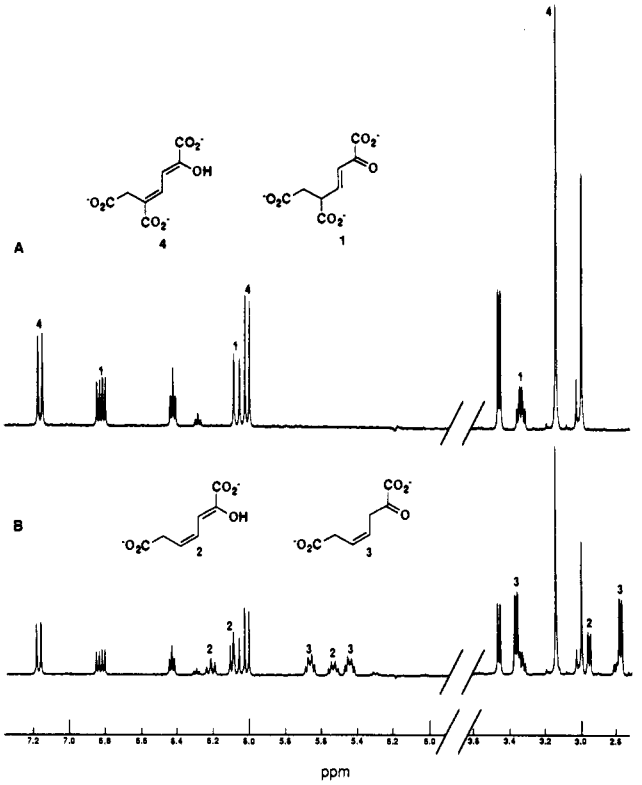



**Figure 1.** $^1H$ NMR (500 MHz, $H_2O$) spectra indicating the generation of **1** and subsequent decarboxylation by COHED to afford **2** and **3**: (A) signals corresponding to **1** and **4**; (B) signals corresponding to **2** and **3**.

These results demonstrate that the appearance of **3** in a mixture of COHED and **1** results predominantly from the enzymatic ketonization of the dienol intermediate **2**. These observations are best explained by the mechanism shown in Scheme II. The instability of enol intermediates in other metal-dependent $\beta$-decarboxylases hampers their isolation and subsequent examination for competency as intermediates in the overall reaction.[4] In

(8) NMR spectra are recorded on a Bruker AM 500-MHz spectrometer. Spectra are recorded in 100% $H_2O$ using a composite pulse selective presaturation of the water signal with a 2-s presaturation interval. Chemical shifts are standardized to the residual $H_2O$ resonance at 4.7 ppm. Figure 1A is generated by the addition of CHMI (6 $\mu$g; 9 units) to a solution of buffer (100 mM $Na_2HPO_4$, 0.6 mL) containing **4** (4.5 mg) which has been dissolved in 10 $\mu$L of DMSO-$d_6$. The addition of **4** to the buffer adjusts the pH to 6.5 and makes the concentration of **4** approximately 35 mM. **1**: $^1H$ NMR ($H_2O$) $\delta$ 2.25 (1 H, dd, $J$ = 8.7, 17 Hz, H-6), 2.49 (1 H, dd, $J$ = 6.5, 17 Hz, H-6), 3.33 (1 H, dd, H-5), 6.06 (1 H, d, $J$ = 17.4 Hz, H-3), 6.84 (1 H, dd, $J$ = 8.7, 17.4 Hz, H-4). **4**: $^1H$ NMR ($H_2O$) $\delta$ 3.14 (2 H, s, H-6), 6.02 (1 H, d, $J$ = 13 Hz, H-3), 7.17 (1 H, d, $J$ = 13 Hz, H-4). The other signals present correspond to the *E* and *Z* isomers of 5-carboxy-2-oxo-4-heptene-1,7-dioate, which result from the nonenzymatic decay of **4**.

(9) Figure 1B is generated by the simultaneous addition of COHED (4.2 units) and CHMI (9 units) to the buffer solution containing **4** as described above.[8] The assignments for **2** were established by comparison to the authentic compound.[11] **2**: $^1H$ NMR ($H_2O$) $\delta$ 2.95 (2 H, d, $J$ = 8.7 Hz, H-6), 5.52 (1 H, dt, $J$ = 10.7 Hz, H-5), 6.09 (1 H, d, $J$ = 12.5 Hz, H-3), 6.22 (1 H, dd, H-4). **3**: $^1H$ NMR ($H_2O$) $\delta$ 2.78 (2 H, d, $J$ = 8.7 Hz, H-6), 3.36 (2 H, d, $J$ = 8.7 Hz, H-3), 5.43 (1 H, dt, $J$ = 10.7 Hz, H-4), 5.65 (1 H, dt, $J$ = 10.7 Hz, H-5).

(10) The $^1H$ NMR spectra following the decarboxylation of **1** by COHED indicate that **2** accumulates in solution. At first glance, this result suggests that COHED catalyzes an unusual two-stage process in which the enzyme converts **1** to **2**, releases the dienol into solution, rebinds the dienol, and catalyzes the reaction of **2** to **3**. It is more likely that the enzyme follows the simpler proposed mechanistic route shown in Scheme II, and the accumulation of **2** in solution results predominantly from the facile buffer-catalyzed (100 mM $Na_2HPO_4$) enolization of **3** after it is released from the enzyme. Previous work on related dienols and results herein[13] demonstrates that phosphate buffer greatly facilitates the interconversion of the dienol and $\beta,\gamma$-enone: Whitman, C. P.; Aird, B. A.; Gillespie, W. R.; Stolowich, N. J. *J. Am. Chem. Soc.* **1991**, *113*, 3154–3162.

(11) The isolation of **2** is described elsewhere:[1,2] $^1H$ NMR (250 MHz, $CD_3OD$) $\delta$ 3.15 (2 H, d, $J$ = 8.3 Hz, H-6), 5.61 (1 H, dt, $J$ = 8.3 Hz, H-5), 6.23 (1 H, d, $J$ = 12.5 Hz, H-3), 6.43 (1 H, dd, $J$ = 10.4 Hz, H-4); $^{13}C$ NMR (500 MHz, $CD_3OD$) $\delta$ 33.3 (C-6), 106.1 (C-3), 123.8 (C-4), 124.9 (C-5), 142.4 (C-2), 165.7 (C-1), 172.2 (C-7).

(12) If the previously proposed mechanism is operative,[1,2,6] nonenzymic ketonization of **2** to **3** is the observed result.

(13) Initially, **2** is protonated almost exclusively at C-3 to give **3**. After 30 min, it is estimated from the kinetic analysis[14] that the solution contains 30.6% **2** and 69.4% **3**. While this result is not in accord with the $^1H$ NMR spectrum acquired at 20 min (Figure 1B), the spectra acquired at later time intervals are in good agreement with the kinetic analysis. The $\alpha,\beta$-enone, 2-oxo-3-heptene-1,7-dioate ($\lambda_{max}$ = 232 nm), is the predominant product (>95%) at equilibrium. It is estimated that after 8 h, 10% of the mixture of **2** and **3** is converted to the $\alpha,\beta$-isomer in 20 mM $NaH_2PO_4$ (pH 7.23).

(14) Three observations indicate that the conversion of **2** to **3** is enzyme-catalyzed. First, increasing the concentration of COHED increases the rate of catalysis. Second, the addition of a comparable amount of enzyme buffer (20 mM $NaH_2PO_4$, pH 7.3; 5 mM $MgCl_2$) without the enzyme does not appreciably accelerate the rate of decay of **2**. Finally, a comparison of $k_{cat}$ for **2** (81 $s^{-1}$) to a preliminary estimate of the uncatalyzed rate of decay ($k_0$) of **2** ($4.9 \times 10^{-3}$ $s^{-1}$) shows about a 16 500-fold rate enhancement. The ketonization of **2** ($\lambda_{max}$ = 276 nm) in aqueous phosphate buffer (0.002–0.05 M) was monitored at pH values 6.50, 7.00, and 7.50 ($\mu$ = 0.2, NaCl, 0.5% methanol), and the data were fit to a single exponential equation. Experiments are underway to determine the stereochemistry of ketonization of **2** to **3** in $^2H_2O$.

(15) $^1H$ NMR (250 MHz, $CD_3OD$) $\delta$ 2.50 (2 H, brd m, H-3), 3.10 (2 H, d, $J$ = 5.5 Hz, H-6), 4.18 (1 H, dd, H-2), 5.68 (2 H, brd m, H-4 and H-5).

addition to elucidating the mechanism of COHED, our results provide strong support for the existence of an enol intermediate in metal-dependent β-decarboxylase reactions.

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## Stereochemistry of a Cyclohexyllithium Reagent. A Case of Higher Configurational Stability in Strongly Coordinating Media[1]

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The use of configurationally defined organolithium reagents in ether solvents has only been possible for the exceptional cases of vinyl,[2] cyclopropyl,[3] α-alkoxyalkyl,[4] and more recently α-selenoalkyl,[1a,5a] and α-aminoalkyl[6] lithium reagents.[7-9] Although early studies by Letsinger[10] and Curtin[11] showed that even secondary alkyllithium reagents lacking such structural features can be prepared in optically active form if conditions are mild and a hydrocarbon solvent is used, they made the discouraging observation that the *rate of racemization was greatly accelerated by small amounts of ether*. Higher configurational lability of normally isomerically stable vinyllithium[2b,c,11] and cyclopropyllithium reagents[12] in the presence of polar solvents has also been reported.

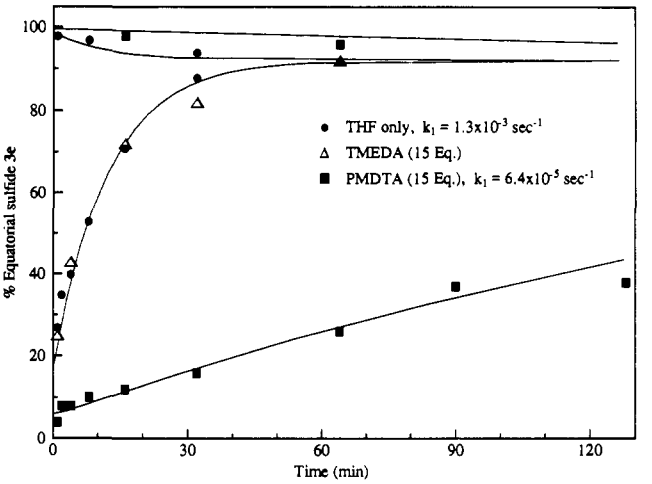
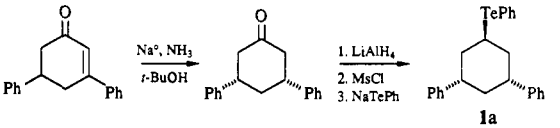



**Figure 1.** Formation, equilibration, and trapping of axial and equatorial lithium reagents **2a** and **2e** formed from **1a** and **1e**. The points are the ratios of isolated methyl sulfides **3a** and **3e**. The lines are best fit pseudo-first-order calculated ratios, using the rate constants shown ([**1**] = 36 mM).

We report here that axial and equatorial cyclohexyllithium reagents[8a] can be formed with high isomeric purity *in THF* and maintain configurational integrity long enough for laboratory time scale trapping experiments. We chose the lithium/tellurium exchange reaction[13] for this study, since it is among the fastest of all Li/M exchanges (only slightly slower than the Li/I[1b,d]), and tellurides have advantages over iodides in ease of preparation and lower reactivity in α- and β-elimination.




**1a**

The axial and equatorial isomers of 1-(phenyltelluro)-*cis*-3,5-diphenylcyclohexane (**1**) were easily prepared from the corresponding alcohols by mesylation and $S_N2$ substitution with sodium phenyltellurolate.[14] Treatment of **1a** with at least 3 equiv of *sec*-butyllithium resulted in fast (<30 s) cleavage of both C–Te bonds at −78 °C to give solutions of predominantly the axial lithium reagent **2a**, as indicated by trapping with dimethyl disulfide to form **3a** and **3e** in combined yields of 80% or better. Di-*sec*-butyl telluride and phenyllithium were also formed, and some unreacted *sec*-butyllithium remained. Apparently inductive withdrawal by the phenyl substituents provides some stabilization of **2** relative to *sec*-butyllithium. However, if less than 3 equiv of *sec*-butyllithium was used, significant amounts of 1-*sec*-butyltelluro-3,5-diphenylcyclohexane remained in the reaction mixture.

The solution of **2a** formed in this way isomerized to **2e** with a half-life of ≈9 min at −78 °C, reaching the equilibrium mixture of 8/92 **2a**/**2e** in 1 h.[8a] The equatorial telluride **1e** similarly gave

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(14) The 3,5-diphenylcyclohexyl system is well suited for these studies, since, in contrast to the 4-*tert*-butylcyclohexyl system, 99% or better isomerically pure materials can routinely be obtained by crystallization, and structure determination by NMR is easy and reliable.[1a]

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