Kinetic and Chemical Mechanisms of Homocitrate Synthase from Thermus thermophilus*

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The homocitrate synthase from Thermus thermophilus (TtHCS) is a metal-activated enzyme with either Mg$^{2+}$ or Mn$^{2+}$ capable of serving as the divalent cation. The enzyme exhibits a sequential kinetic mechanism. The mechanism is steady state ordered with α-ketoglutarate (α-Kg) binding prior to acetyl-CoA (AcCoA) with Mn$^{2+}$, whereas it is steady state random with Mg$^{2+}$, suggesting a difference in the competence of the E$^{2+}$Mnα-Kg:AcCoA and E$^{2+}$Mgα-Kg:AcCoA complexes. The mechanism is supported by product and dead-end inhibition studies. The primary isotope effect obtained with deuterioacetyl-CoA (AcCoA$^{-2}$H$_3$) in the presence of Mg$^{2+}$ is unity (value 1.0) at low concentrations of AcCoA, whereas it is 2 at high concentrations of AcCoA. Data suggest the presence of a slow conformational change induced by binding of AcCoA that accompanies deprotonation of the metal group of AcCoA. The solvent kinetic deuterium isotope effect is also unity at low AcCoA, but is 1.7 at high AcCoA, consistent with the proposed slow conformational change. The maximum rate is pH independent with either Mg$^{2+}$ or Mn$^{2+}$ as the divalent metal ion, whereas $V/K_{\alpha\text{-Kg}}$ (with Mn$^{2+}$) decreases at low and high pH giving $pK$ values of about 6.5 and 8.0. Lysine is a competitive inhibitor that binds to the active site of TtHCS, and shares some of the same binding determinants as α-Kg. Lysine binding exhibits negative cooperativity, indicating cross-talk between the two monomers of the TtHCS dimer. Data are discussed in terms of the overall mechanism of TtHCS.

The α-aminoadipate pathway for lysine biosynthesis is nearly unique to higher fungi, including human and plant pathogens and euglenoids; an exception is the thermophilic bacterium Thermus thermophilus. The α-aminoadipate pathway is comprised of eight enzymatic reactions catalyzed by seven enzymes. Homocitrate synthase (HCS) catalyzes the first and regulated step in this pathway, the condensation of acetyl-CoA (AcCoA) and α-ketoglutarate (α-Kg) to give homocitrate and coenzyme A (CoASH) (Scheme 1). As a result, it is a potential target for the development of new antifungals against human pathogens such as Candida albicans and Aspergillus fumigatus.

The best studied of the homocitrate synthases is that from Saccharomyces cerevisiae (4–8). The homocitrate synthase from Saccharomyces cerevisiae (ScHCS) is a Zn-metalloenzyme (8). The ScHCS and that from C. albicans (CaHCS) are not particularly stable as isolated; but they are stable for at least 2 months at 4°C in the presence of the additives, guanidine hydrochloride, α-cyclodextrin, and (NH$_4$)$_2$SO$_4$ (5). In the absence of the additives, however, the enzymes rapidly lose activity, and this limits the type of experiments that can be carried out, especially structural studies (5).

An ordered kinetic mechanism has been proposed for ScHCS and CaHCS with α-Kg binding prior to AcCoA (4). Lysine is a feedback inhibitor of ScHCS, and is competitive versus α-Kg. Inhibition was thought to result from binding to an allosteric site, stabilizing a less active conformer of the enzyme. A chemical mechanism for ScHCS has been proposed in which α-Kg is bound to the active site zinc via its α-carboxylate and α-oxo groups, in the vicinity of the thioester of AcCoA. A general base accepts a proton from the methyl group of AcCoA as it attacks the thioester of AcCoA, and a general acid protonates the carbonyl of α-Kg in the formation of homocitryl-CoA. The general acid then acts as a base in the deprotonation of Zn-OH$_2$ in the hydrolysis of homocitryl-CoA to give homocitrate and CoA (8). Isotope effect data suggest the product of the condensation of AcCoA and α-Kg is the alkoxide, which is then protonated by a general acid in the next step (8).

Multiple sequence alignment of the residues around the active site of HCS show conservation of ScHCS residues Glu-155, His-309* (* indicates a residue from the other monomer), and Tyr-320 in TtHCS, isopropylmalate synthase, and citramalate synthase (CMS). Site-directed mutagenesis indicates that Glu-155 and His-309* form the catalytic dyad that is proposed to deprotonate the methyl of AcCoA and Tyr-320 may be involved in orienting the reactants and/or the catalytic dyad for catalysis (6).

In most bacteria, lysine is synthesized from aspartic acid via the diaminopimelate pathway. However, in T. thermophilus, lysine is synthesized via the α-aminoadipate pathway. Crystal structures of TtHCS have recently been solved (10), but a detailed study of the kinetic and/or chemical mechanism of TtHCS has not been carried out. In this article, the TtHCS has been subjected to initial rate and isotope effect studies, and the pH dependence of kinetic parameters has been measured. Based on these results kinetic and chemical mechanisms are proposed for the TtHCS enzyme and comparisons are made to those proposed for ScHCS.

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2. The abbreviations used are: HCS, homocitrate synthase; Hc, homocitrate; AcCoA, acetyl-CoA; AcCoA$^{-2}$H$_3$, deuterioacetyl-CoA; α-Kg, α-ketoglutarate; DCPIP, 2,6-dichlorophenolindophenol; Taps, N-tris(hydroxymethyl)methyl-3-amino-1-propanesulfonic acid; BisTris, 2,3-bis-(2-hydroxyethyl)-amino-2-(2-hydroxyethyl)-1-propanediol; CMS, citramalate synthase; OG, oxa glyco; Hepes, N-(2-hydroxyethyl)piperazine-N-(2-ethane sulfonic acid).
3. V. P. Kumar, unpublished data.
MATERIALS AND METHODS

Chemicals—α-Ketoglutarate, AcCoA, CoA, 2,6-dichlorophenolindophenol (DCPIP), oxaloacetate, oxalate, EDTA, and Chelex 100 were from Sigma. N-Oxalylglycine was obtained from Frontier Scientific. Imidazole, KCl, CaCl₂, MgCl₂, MnCl₂, ZnCl₂, CoCl₂, and NiCl₂ were from Fisher Scientific. The buffers Taps, Hepes, BisTris, and Mes were from Research Organics, whereas the nickel-nitrilotriacetic acid resin was purchased from 5Prime. MagicMedia used for cell growth was purchased from Invitrogen. Perdeuterioacetic anhydride (98 atom% D) and D₂O (99 atom% D) were purchased from Cambridge Isotope Laboratories, Inc. Deuterioacetyl-CoA was prepared from CoA and perdeuterioacetic anhydride according to the method of Simon and Shemin (11).

Cell Growth and Expression—Histidine-tagged homocitrate synthase from _T. thermophilus_ was previously cloned into the pET26b⁺ (Novagen) vector (12). The _Tt_HCS clone was transformed into _Escherichia coli_ BL21(DE3) RIL star cells and the culture was grown in MagicMedia containing chloramphenicol (34 μg/ml) and kanamycin (10 μg/ml) at 37 °C overnight. Cells were lysed by sonication in 50 mM Hepes, pH 7.5, containing 30 mM KCl, 5 mM imidazole, and enzyme was purified using the nickel-nitrilotriacetic acid resin, with elution at 360 mM imidazole. The enzyme was >95% pure by SDS-PAGE. The enzyme was stored at 4 °C in the elution buffer.

Enzyme Assay—HCS activity was measured using the DCPIP assay developed previously (5), monitoring the decrease in absorbance at 600 nm as DCPIP is reduced by CoASH. Reactions were carried out in quartz cuvettes with a path length of 1 cm and a final volume of 0.5 ml containing 50 mM Hepes, pH 7.5, 0.1 mM DCPIP, and variable concentrations of α-Kg and AcCoA. The concentrations of AcCoA and DCPIP were estimated spectrophotometrically, using the following extinction coefficients: AcCoA, ε₂₅₀ = 16,400 M⁻¹ cm⁻¹; DCPIP, ε₆₀₀ = 19,100 M⁻¹ cm⁻¹. Assays were carried out at 25 °C and reactions were thermally equilibrated to complete the reaction between the small amount of CoA in the AcCoA solution and DCPIP before adding the enzyme to the reaction mixture.

Divalent Metal Ion Specificity—To determine the effect of metal ions on TtHCS, apoenzyme was prepared by dialyzing the isolated enzyme against 50 mM Hepes pH 7.5 containing 100 mM EDTA, followed by dialysis against 50 mM Hepes pH 7.5 buffer. All reagents for dialysis or enzyme assay were passed through Chelex resin to remove metal ions. All reagents used in these experiments were stored in polypropylene bottles and tubes. The effect of the presence of divalent cations such as Mg²⁺, Mn²⁺, Ca²⁺, Ni²⁺, Zn²⁺, and Co²⁺ and monovalent cations such as Na⁺ and K⁺ on the activity of _TtHCS_ was tested by initial velocity measurements. The enzyme assay was carried out as described above.

Initial Velocity Studies—To determine the kinetic mechanism of _TtHCS_, initial velocity patterns were obtained by measuring the initial rate as a function of AcCoA concentration at different fixed concentrations of α-Kg (or oxaloacetate). The reaction typically consisted of 50 mM Hepes, pH 7.5, 0.1 mM DCPIP, 200 μM MgCl₂, 1 μM _TtHCS_, and variable concentrations of α-Kg and AcCoA. Similar initial velocity patterns were also obtained with 200 μM MnCl₂ as the metal ion in place of MgCl₂.

Inhibition patterns were obtained by measuring initial rates at different concentrations of one reactant at a fixed concentration of the other (AcCoA was maintained at 50 μM, whereas α-Kg was maintained at 1 mM) in the absence and presence of inhibitor. Inhibition by CoASH, made use of the disappearance of absorbance at 232 nm (reflecting the thioester bond of AcCoA) using an ε₂₃₂ = 4500 M⁻¹ cm⁻¹ (4). The apparent inhibition constant for lysine was also measured as a function of pH from 5.9 to 8.8 at fixed concentrations of α-Kg (100 μM) and AcCoA (50 μM) in the presence of MgCl₂ (200 μM).

pH Studies—It is important to confirm that the enzyme is stable for the duration of the assay in the pH range that is being tested. To test this, the enzyme was preincubated at the given pH and aliquots were removed at regular time intervals and assayed at pH 7.5, where the enzyme is known to be stable. The pH dependence of V and V/K was obtained by measuring the initial rate as a function of one substrate, maintaining the other at a fixed concentration (α-Kg, 5 mM; AcCoA, 5 μM). Initial velocity patterns were obtained over the pH range of 6–9 and Km values were estimated for both α-Kg and AcCoA (not done for Mg²⁺). This information determined the concentration range of substrates to be used over the entire pH range. Buffers were maintained at 50 mM concentration in the following pH ranges: BisTris, 6.0–7.0; Hepes, 7.0–8.0; Taps, 8.0–9.0. The pH of the reaction mixture was recorded before and after the reaction with little difference noted. The data were then analyzed by plotting logV or logV/K versus pH.

Isotope Effects—Primary deuterium kinetic isotope effects were measured by direct comparison of initial velocities, where AcCoA-d₄ was used as the deuterated substrate and α-Kg was
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fixed at saturating concentration (400 μM). Solvent deuterium isotope effects were obtained by direct comparison of initial rates as a function of α-Kg at different fixed levels of AcCoA in H2O and D2O over the pH(D) range 6–9 (around the pH independent region of the V (or V/K) pH rate profile). For these studies, all reagents including buffers, substrates, and DCPIP were first dissolved in a small amount of D2O, then lyophilized overnight to remove H2O and re-dissolved in the required volume of D2O. The pH(D) was adjusted using KOD or DC1. The initial rate was measured in H2O and D2O (18).

Data Processing—Data were fitted to the appropriate rate equation using the EnzFitter program from BIOSOFT, Cambridge, UK, and the programs developed by Cleland (14). In some cases data could not be fitted by the computer program, and kinetic parameters were obtained from graphical analysis. Initial velocity data with MnCl2 were fitted to Equation 1. Competitive and uncompetitive inhibition data were fitted using Equations 2 and 3. Isotope effect data were fitted to Equation 4.

\[
v = \frac{V_{AB}}{K_B(1 + \frac{B}{K_{ni}}) + K_A + AB} \quad (\text{Eq. 1})
\]

\[
v = \frac{V_A}{K_o(1 + \frac{1}{K_{oi}}) + A} \quad (\text{Eq. 2})
\]

\[
v = \frac{V_A}{K_o + A(1 + \frac{1}{K_{oi}})} \quad (\text{Eq. 3})
\]

\[
v = \frac{V_A}{K_o(1 + F_{E_{VIK}}) + A(1 + F_{E_V})} \quad (\text{Eq. 4})
\]

In Equations 1–4, v and V are initial and maximum velocities, respectively, and 1 and 2 are substrate and inhibitor concentrations, K and Ki are Michaelis constants for substrates A and B, respectively, whereas Kii and Kii are slope and intercept inhibition constants, respectively. In Equation 4, F is the atom fraction of deuterium in the labeled component, whereas E and E are the isotope effects minus 1 on the given parameter.

Data for lysine inhibition were fitted to Equation 5 (13),

\[
\log v = \log \left( \frac{V[dA + A^2]}{c + bA + A^2} \right) \quad (\text{Eq. 5})
\]

where b, c, and d are defined in Equations 6–8.

\[
b = d + \frac{S_{Lo}}{\text{INT}_o} \quad (\text{Eq. 6})
\]

\[
c = d \left[ \frac{S_{Lo}}{\text{INT}_o} \right] \quad (\text{Eq. 7})
\]

\[
d = \frac{S_{Lo} - S_{Li}}{\text{INT}_o - \text{INT}_s} \quad (\text{Eq. 8})
\]

The parameters SL and S refer to the slope of the double reciprocal plot with the concentration of A tending toward 0 and infinity, whereas INT and INT refer to the intercept of the double reciprocal plot extrapolating the linear asymptote at low A to the ordinate and the ordinate intercept at infinite A, respectively.

The pH dependence of V/KAcCoA was fitted to Equation 9, whereas data for the pH dependence of the apparent KiLys was fitted to Equation 10.

\[
\log y = \log \left[ \frac{C}{1 + \frac{H}{K_i} + \frac{K_2}{H}} \right] \quad (\text{Eq. 9})
\]

\[
\log y = \log \left[ \frac{Y_c + Y_p \left( 1 + \frac{10^{-9.5}}{H} \right)}{1 + \frac{10^{-9.5}}{H}} \right] \quad (\text{Eq. 10})
\]

In Equation 9, y is the observed value of V/K at any pH, C is the pH-independent value of y, H is the hydrogen ion concentration, and Ki and K2 are the acid dissociation constants of functional groups required in a given protonation state on enzyme or substrate for optimal binding and/or catalysis. In Equation 10, y is the value of 1/KKiLys at any pH. Y and Y are the pH independent values of 1/KKiLys at low and high pH, respectively, and the value 10^{-9.5} is the acid dissociation constant for the α-amine of lysine.

RESULTS

Divalent Metal Ion Specificity—As isolated, TtHCS has metal ions bound. Treatment with EDTA removed the bound metal ions. The dependence of rate on added metal ion was measured with the EDTA-treated TtHCS. In the absence of any exogenous metal ion, apoenzyme had negligible activity (Fig. 1). The enzyme could be reactivated by the addition of Mg2+ or Mn2+, whereas in the presence of Ni2+ or Zn2+, no detectable activity was observed (Fig. 1). A slight activity was observed in the presence of Co2+, but the stock used was only 97% pure and had significant contamination with other divalent metal ions including Mg2+ and Mn2+. Reactivation to a lesser extent was found with addition of the monovalent cations, Na+ and K+ (data not shown). It is likely that activation by Na+ and K+ results from contaminating Mg2+. A saturating concentration of Mn2+ or Mg2+ is 200 μM. Dialysis of the magnesium-recon-
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2.5 \( K_{\text{AcCoA}} \cdot K_i = (\text{app } K_i)/(1 + A/K_a) \) (Fig. 4B). At high concentrations of CoASH, curved double reciprocal plots were observed (Fig. 4A). Data are summarized in Table 3.

Oxalylglycine (OG), a \( \alpha \)-Kg analog, is competitive versus \( \alpha \)-Kg, and \( K_{i,\text{OG}} \) is independent of the concentration of AcCoA (data not shown). Oxaloacetate was a slow alternative substrate analog of \( \alpha \)-Kg, with a \( V/E_q \) of 0.0230 ± 0.0006 s⁻¹ and \( V/K_{\text{OAA}}, E_q \) of 0.065 ± 0.019 M⁻¹ s⁻¹ (data not shown). Although the maximum rate with OAA is only 5-fold lower than that with \( \alpha \)-Kg, \( V/K_{\text{OAA}}, E_q \) is 3.5 × 10⁴-fold lower than \( V/K_{\alpha \text{-Kg}}, E_q \), suggesting OAA binding is about 7000-fold weaker than \( \alpha \)-Kg. Other analogs of \( \alpha \)-Kg, including oxalate showed no significant inhibition at 1 mM with \( \alpha \)-Kg fixed at 1.5 \( K_m \). Malonyl-CoA and pantothenate, analogs of AcCoA/CoASH, gave no inhibition in 1 mM concentration, with AcCoA fixed at \( K_m \) and \( \alpha \)-Kg fixed at a saturating concentration (1 mM). Data are summarized in Table 3.

Lysine, the final product of the aminoadipate pathway, exhibits feedback inhibition of HCS (6). Lysine is a competitive inhibitor versus \( \alpha \)-Kg up to a concentration of 1 mM (Fig. 5A). Inhibition by lysine is nonlinear, as shown by a Dixon plot of \( 1/v \) versus lysine at fixed concentrations of \( \alpha \)-Kg (16 \( K_{\alpha \text{-Kg}} \)) and AcCoA (2.5 \( K_{\text{AcCoA}} \)) (Fig. 5B). Data are suggestive of negative cooperativity in the binding of lysine. A fit of the data to Equation 4 gives well defined \( K_i \) values for binding the first and second molecules of lysine (Table 3). Inhibition by lysine is also competitive versus \( \alpha \)-Kg in the high lysine concentration range, however, the double reciprocal plots are concave upward at high lysine concentration (Fig. 5C). Data suggest that the presence of high concentrations of lysine induce positive cooperativity in binding \( \alpha \)-Kg, as observed for CoASH.

Primary Substrate Deuterium Kinetic Isotope Effects—Primary substrate deuteration kinetic isotope effects were measured with Mg²⁺ as the divalent metal ion by direct comparison of initial rates at pH 7.5 with acetyl-\( d_1 \) CoA as the labeled substrate. An isotope effect of unity was obtained at low AcCoA concentration, but a kinetic isotope effect of about 2 is observed at high concentrations of AcCoA (Fig. 6).

Solvent Deuterium Kinetic Isotope Effects—Solvent deuteration kinetic isotope effects were measured in the presence of 200 \( \mu \)M Mg²⁺ at pH(D) 7.5, in the pH independent region of the pH rate profiles. Initial velocity patterns were obtained by measuring the initial rate at varying \( \alpha \)-Kg concentrations and different fixed levels of AcCoA in H₂O and D₂O (Fig. 7, A and B). Slope and intercept replots derived from the primary plot are shown in Fig. 7, C and D. Solvent isotope effects were unity at low concentrations of AcCoA, but were finite at high concentrations of AcCoA, with estimated values for \( ^{12}O/V \) of 1.7 and \( ^{12}O/V/K_{\text{AcCoA}} \) of 2.8 (Fig. 7E).

\( pH \) Dependence of Kinetic Parameters—The \( pH \) dependence of kinetic parameters provides information on the optimal protonation state of functional groups on enzyme and/or substrate for binding and/or catalysis. To be certain that the kinetic mechanism of the enzyme does not change with \( pH \) and to obtain estimates of \( K_m \) values for both substrates as a function
of pH, initial velocity patterns were obtained with α-Kg and AcCoA concentrations varied at each pH. In the presence of MnCl₂ with AcCoA as the varied substrate, V was pH independent, whereas V/Kₐ-Kg decreased at low pH with a slope of 1 giving a pKₐ of about 6.5 and at high pH with a slope of −1, giving a pKₐ of about 8.0 (Fig. 8). Vₘₐₓ with Mg²⁺ as the divalent metal ion is also pH independent (data not shown). The pH dependence of V/Kₐ-AcCoA was not determined because of the large uncertainty in the values (Table 2), estimated from data similar to those in Fig. 3.

An apparent Kᵣ for lysine inhibition was measured by a Dixon plot for the low lysine concentration range (Fig. 5B). Over the pH range studied, Kᵣₜₐₜₛ decreased from a constant value below pH 6.0 to another constant value above pH 8.8 (Fig. 5C). A value of 9.5 was assumed for the pKᵣ obtained from the change in Kᵣ (see explanation under “Discussion”), and data were fitted to Equation 10. The pH independent values of Kᵣₜₐₜₛ at high and low pH are 1.9 ± 0.4 mM and about 8 mM, respectively, and the pKᵣ of the α-amine of lysine when it is bound to enzyme is 5.9 ± 0.2.

DISCUSSION

Divalent Metal Ion Specificity—The apoenzyme of TtHCS can be activated by Mg²⁺ and Mn²⁺, and is thus likely a magnesium-dependent metal-activated enzyme. Although Mn²⁺ also activates the apoenzyme to the same extent as Mg²⁺, the
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FIGURE 4. Inhibition by CoA at variable concentrations of α-ketoglutarate. Initial rates were obtained as a function of α-Kg concentration as shown with AcCoA fixed at 50 μM (A). The concentrations of CoASH used are as follows: 0 (■), 5 (○), 50 (▲), 100 (×), and 200 (●) μM. B, additional data for the concentration range shown in the dotted box in A. The enzyme concentration was maintained at 1 μM. Data at high concentration of α-Kg (>100 mM) were fit to Equation 3.

TABLE 3
Summary of inhibition studies

| Variable substrate | Fixed substrate | Inhibitor | $K_{ia} \pm S.E.$ | $K_{ib} \pm S.E.$ | Pattern |
|--------------------|-----------------|-----------|-----------------|-----------------|---------|
| AcCoA              | α-Kg            | CoASH     | 0.026 ± 0.01    | 0.11 ± 0.03     | C       |
| α-Kg               | AcCoA           | CoASH     | 0.9 ± 0.1       |                  | C       |
| α-Kg               | AcCoA           | OG        |                  |                  | C       |
| α-Kg               | AcCoA           | Lysine    | Site 1, 0.23 ± 0.06 Site 2, 56 ± 14 | |

total cellular levels of Mn$^{2+}$ are generally in the micromolar range (21, 22, 29) and it is thus unlikely that Mn$^{2+}$ is the physiologic activator. Interestingly, the initial velocity patterns obtained with Mg$^{2+}$ and Mn$^{2+}$ as the activator are different (see below), suggesting that the size of the metal ion and its coordination geometry may contribute to this difference. TtHCS differs from the ScHCS, which is a Zn-metalloenzyme; Zn$^{2+}$ does not activate TtHCS. A recent crystal structure (10) suggests Co$^{2+}$ is bound to TtHCS in an apparent octahedral geometry with three ligands from enzyme, the side chains of Glu-13, His-197, and His-195, the C1-carboxylate and C2-oxo groups of α-Kg, and a water molecule. However, these authors also showed significant activity of the enzyme in the presence of Co$^{2+}$, whereas no significant activation of TtHCS was seen with Co$^{2+}$ in these studies. There was not enough information provided to determine how metal ion solutions were treated in the previous study (10), and activity could result from contaminating metal ions in the Co$^{2+}$ solution.

Kinetic Mechanism—The kinetic mechanism depends on the divalent metal ion and keto acid substrate used in the TtHCS reaction. In the presence of Mn$^{2+}$, an initial velocity pattern was obtained that is near parallel at low concentrations of AcCoA, suggesting a $K_{iα-Kg}/K_{α-Kg}$ ratio that is low. Under these conditions the $K_{ia}K_{ib}$ term in the sequential rate equation ($v = (V_{AB})/(K_{iα-Kg} + K_{α-Kg} + K_{iA} + AB)$) becomes negligible and the rate equation reduces to the same as that for a ping-pong mechanism. The replot of slope versus 1/AcCoA is concave upward (Fig. 2). However, the intercept replots is linear indicating substrate inhibition by AcCoA that is competitive versus α-Kg. Thus, the kinetic mechanism appears to be steady state ordered with α-Kg binding prior to AcCoA (Scheme 2) with a dead-end $E$AcCoA complex allowed at high concentrations of AcCoA, described by Equation 1. It is interesting to note that the substrate inhibition constant for AcCoA is essentially equal to its $K_{iα-Kg}$, 10 ± 5 μM compared with 27 ± 4 μM. At low, sub-saturating α-Kg, the $E$AcCoA complex will form at concentrations of AcCoA around 10 μM, decreasing the overall amount of productive enzyme, whereas at high saturating concentrations of α-Kg, the reaction proceeds toward product at the same concentration of AcCoA. Thus, with Mn$^{2+}$ as the metal ion activator, α-Kg determines the rate of the TtHCS reaction.

The mechanism differs considerably in the presence of Mg$^{2+}$. The initial velocity pattern obtained varying α-Kg at different fixed levels of AcCoA intersects to the left of the ordinate at low AcCoA, but slope and intercept replots exhibit curvature at high AcCoA concentrations (Fig. 3A, insets). Note, however, that in the primary plot of 1/v versus 1/[AcCoA], the pattern exhibits near parallel lines at low AcCoA (Fig. 3B) with the extrapolated curves at high AcCoA intersecting the ordinate at a finite value. Data are indicative of a steady state random kinetic mechanism (Scheme 3). The near parallel lines at low AcCoA suggest, as for data obtained with Mn$^{2+}$, a low $K_{iα-Kg}/K_{α-Kg}$ ratio. The pathway to which this condition applies is that for α-Kg adding prior to AcCoA the same pathway that is productive with Mn$^{2+}$ as the divalent metal ion. Limits of the kinetic parameters were estimated as discussed under “Results” and Table 1. The pathway with α-Kg adding prior to AcCoA is about 3 times faster than the pathway with AcCoA adding prior to α-Kg as shown by the ratio of the maximum rates obtained at low and high AcCoA. The $K_{iα-Kg}$ values for α-Kg and AcCoA are...
not significantly different for the two pathways, and thus the $V/K$ values for $\alpha$-Kg and AcCoA mirror changes in $V$. It is interesting to note that the pathway with AcCoA binding to free enzyme is productive with $\text{Mg}^{2+}$ as the divalent metal ion, whereas AcCoA exhibits substrate inhibition binding to $E$ with $\text{Mn}^{2+}$ as the divalent metal ion. (A small amount of activity at high AcCoA cannot be ruled out.) Data certainly suggest something different about the active site with $\text{Mg}^{2+}$ and $\text{Mn}^{2+}$ bound.

Product inhibition by CoASH provides additional information on the kinetic mechanism of TtHCS. In the presence of $\text{Mg}^{2+}$, or $\text{Mn}^{2+}$, CoASH was competitive against AcCoA suggesting the binding of CoASH to the same enzyme form(s) as AcCoA, free enzyme, and the $E\alpha$-$\text{Kg}$ complex (data not shown). Inhibition by CoASH versus $\alpha$-Kg was uncompetitive (Fig. 4B). In addition, at high concentrations of CoASH, curved double reciprocal plots were observed with $\alpha$-Kg varied (Fig. 4A). Data suggest induction of positive cooperativity of $\alpha$-Kg binding, and this will be discussed below when lysine inhibition is considered. The CoASH inhibition patterns differ from those found for ScHCS, which exhibits noncompetitive inhibition by CoASH versus AcCoA, and uncompetitive inhibition versus $\alpha$-Kg (4). Data for the yeast enzyme were consistent with the ordered addition of a $\alpha$-Kg prior to AcCoA and with binding of CoASH to the $E\alpha$-$\text{Kg}$ and $E$-homocitrate ($E\text{Hc}$) complexes. The CoASH inhibition patterns obtained for the TtHCS are consistent with CoASH binding to $E\alpha$-$\text{Kg}$, but not to the $E\text{Hc}$ complex. Data suggest that release of homocitrate from the $E\text{Hc}$ complex does not contribute to rate limitation of the bacterial enzyme.

The $\alpha$-Kg analog, OG, is competitive as expected, but $K_{i,OG}$ is independent of the concentration of AcCoA, suggesting bind-
Inhibition by Lysine—Lysine is known to inhibit THCS, suggesting end product feedback regulation of the biosynthetic pathway (8). A Dixon plot of the reciprocal rate versus the concentration of lysine is biphasic (Fig. 5), suggesting negative cooperativity of binding lysine. Lysine is a competitive inhibitor versus α-Kg at low concentrations, where it has the highest affinity for enzyme and at high concentrations, where it binds with lower affinity (Scheme 3). The difference in affinity for the two sites is about 250-fold (Table 3). At high concentrations, however, lysine induces positive cooperativity of α-Kg binding, similar to that observed with CoASH. The positive cooperativity is likely a result of the two sites generated by lysine binding, i.e. induced asymmetry in the enzyme dimer in the presence of lysine. By analogy a similar phenomenon likely occurs with CoASH. Because lysine and α-Kg compete for the same site, α-Kg can effectively compete for lysine at much lower concentrations at the low affinity site, but higher concentrations are required to compete for lysine at the high affinity site. This would require that α-Kg competing with lysine at the weak site would decrease the affinity for lysine at the tight site, while increasing its own affinity for that site, i.e. the two ligands have opposing effects.

THCS exists as a homodimer and this, in conjunction with the crystal structure of THCS with lysine bound (Fig. 9B) (10), provides a possible molecular basis for the negative cooperativity of lysine binding. The competitive inhibition suggests, and the lysine-bound structure confirms, that lysine binding is at the active site. In fact, the α-amino and α-carboxylate of lysine occupy the same coordination positions on the active site Mg\(^{2+}\) as the α-oxo and α-carboxylate of α-Kg occupy once bound. The hypothesis is that binding of the first molecule of lysine into the first active site brings about a conformational change in the enzyme such that its affinity for the second active site of the homodimer is reduced, resulting in the second phase observed in the Dixon plot (Fig. 5). Binding of lysine results in dramatic structural changes (10). For example, helix 11 moves by about 44 Å from one side of the protein to the other. In addition, although lysine and α-Kg share some of the same active site binding determinants, there are a number of differences (see “Structure” below). The conformational change differs from the one(s) generated upon binding of reactants (see “Structure” below).

In agreement with the above, and consistent with the structure, is the pH dependence of pK\(_a\) for binding to site 1. Lysine is coordinated to the active site Mg\(^{2+}\) by its α-amino and α-carboxylate. As a result, the α-amino must be neutral for optimum binding. The apparent pK\(_a\) decreases from a constant value at high pH (above pH 9) and goes to a lower constant value at low pH (below pH 5.5). Thus, the group with a pK\(_a\) above 9 that must be unprotonated to coordinate to the metal ion is the α-amino of lysine, which has a pK\(_a\) of about 9.5. A fit of the data to Equation 9 gives an estimate of about 6 for the pK\(_a\) of the α-amino of lysine bound to the active site Mg\(^{2+}\). The pK\(_a\) for lysine changes by more than 3 orders of magnitude as the pH increases with the value above pH 10 reflecting the true pK\(_a\) (2 μM) for lysine with a neutral α-amino binding to enzyme. Lysine
with a protonated α-amine binds 4000-fold weaker, and the pKa of the α-amine decreases by 3.6 pH units, reflecting the much lower proton affinity for lysine bound to the active site in the vicinity of the metal ion.

The negative cooperativity of lysine binding essentially results in decreasing activity of both active sites of the HCS dimer upon occupancy of a single subunit. The physiologic concentration of lysine can reach 5 mM (23–28), a concentration at which the tight site of the TtHCS is saturated (Ki is 230 μM, Table 3). This suggests the activity of TtHCS will depend on the concentration of α-Kg. Although the physiologic concentration will likely not get high enough to allow binding to the loose site, the phenomenon of negative cooperativity in conjunction with the structural data available, aid in determining the mode of lysine binding.

Rate-determining Steps—In the presence of Mg2+ , isotope effect data are complex, but revealing. A substrate deuterium isotope effect of unity is measured with AcCoA-d3, at low concentrations of AcCoA, but a finite primary kinetic isotope effect of about 2 is observed at high AcCoA. Data can be interpreted in terms of the steady state random kinetic mechanism proposed for TtHCS (see “Kinetic Mechanism” above) as observed previously for homoisocitrate dehydrogenase (17).

At low AcCoA, α-Kg binds prior to AcCoA and this is the dominant pathway. The deuterium kinetic isotope effect reflects deprotonation of the methyl of AcCoA, whereas the solvent deuterium kinetic isotope effect reflects protons in flight in the rate-limiting transition state. Both isotope effects are unity for the pathway with α-Kg binding first, indicating deprotonation of the methyl group of AcCoA does not contribute to rate limitation, nor does hydrolysis of the homocitryl-CoA that is produced in the Claisen condensation. As a result, we propose that a conformational change that likely follows the chemical steps limits the overall reaction (see below).

At high AcCoA, where it binds prior to α-Kg, a finite primary kinetic isotope effect of about 2 is observed (Fig. 6). We propose that binding of AcCoA to free enzyme elicits a conformational change that is slower than the one that must occur when AcCoA binds second. The difference between the two is the presence or absence of α-Kg, which can pre-organize the active site for binding of AcCoA. Because an isotope effect is observed
when AcCoA binds first, deprotonation of the methyl group of AcCoA must accompany the conformational change. The solvent deuterium kinetic isotope effects exhibit the same behavior as the substrate kinetic isotope effect, suggesting the conformational change that occurs when AcCoA binds first must have protons in flight in the transition state for the conformational change, giving an apparent solvent effect of about 1.7 (Fig. 7).

If the conformational change elicited by AcCoA binding after α-Kg were slow, one would expect finite primary deuterium and solvent deuterium kinetic isotope effects. Thus, the slow step in the pathway with AcCoA binding after α-Kg likely occurs after the chemical steps, i.e., release of the first product. The interpretation is illustrated in Scheme 4. In the scheme, the mechanism is broken down to show the two pathways. Pathway A is the dominant pathway with α-Kg binding before AccoA, and the slow step is the conformational change that accompanies release of CoA. (Product inhibition by CoA is strictly competitive versus AcCoA and uncompetitive versus α-Kg, consistent with absence of binding of CoA to free enzyme or an E-Hc product complex. By analogy to the forward reaction, release of CoA before Hc is predicted, and data therefore suggest rapid release of Hc.) Pathway B, on the other hand, describes binding of AcCoA before α-Kg, and the slow step in this case is the conformational change elicited upon binding of AcCoA, which includes deprotonation of the methyl of AcCoA.

**pH Dependence of Kinetic Parameters**—The pH dependence of $V/E_t$ is obtained at saturating concentrations of all substrates. The $V$ profile will thus reflect ionizable groups within the enzyme-substrate complex involved in catalysis. The $V/K_E$ pH rate profile reflects ionizable groups, on free substrate and the enzyme form to which it binds, required in an optimum protonation state for binding and/or catalysis. Data for the pH dependence of $V$ and $V/K$ were obtained in the presence of Mn$^{2+}$, however, the difference in kinetic mechanism with Mg$^{2+}$ and Mn$^{2+}$ lies in the competence of the $E$-AcCoA complex. Catalytic and binding groups, and the pH dependence of kinetic parameters for the pathway with α-Kg binding first should be very similar whatever the divalent metal ion used.

With Mn$^{2+}$ as the divalent metal ion, $V$ is pH independent, whereas $V/K_{AcCoA}$ decreases at low and high pH giving pK$\alpha$ values of about 6.5 and 8.0, respectively. (The maximum rate obtained with Mg$^{2+}$ was also pH independent (data not shown).) The pH dependence of $V/K_{AcCoA}$ was not determined because of the large uncertainty in the values (Table 2) estimated from data similar to those in Fig. 3. Data are consistent with the requirement for general base, general acid mechanism, as proposed for the *Saccharomyces* enzyme, ScHCS. By analogy to ScHCS, the base is proposed to accept a proton from the methyl of AcCoA to allow nucleophilic attack on the carbonyl of α-Kg, whereas the acid is proposed to donate a proton to the carbonyl oxygen to form homocitryl-CoA (6). The active site residues of ScHCS and TtHCS are conserved, and will be further described below.

**Structure**—TtHCS is a dimer comprised of a TIM barrel domain and C-terminal small domains (I and II) (10). Small domain II of one monomer is a 3-helix bundle that covers the active site of the other monomer; the active site is on the C-terminal end of the TIM barrel. This arrangement, described as
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**Scheme 3.** Kinetic mechanism proposed for T. thermophilus homocitrate synthase with Mg\(^{2+}\) as the metal ion. A random kinetic mechanism is shown highlighting the conformational change that occurs upon AcCoA binding. The conformations of the enzyme are denoted as \(E\) and \(E^*\). Lysine binds to free enzyme competing with \(\alpha\)-Kg, and generating a dead-end \(E\)-lysine complex.

**Scheme 4.** The two pathways of the random mechanism shown in Scheme 3 are shown. Pathway A is the dominant pathway and exhibits rate-limiting release of CoA. The deuterium and solvent deuterium sensitive steps reflect the conversion of \(E^*\)-\(\alpha\)-Kg:AcCoA to products. Pathway B occurs at high concentrations of AcCoA. The slow step in this case is the conformational change that accompanies AcCoA binding to give the \(E^*\)-AcCoA complex. This step exhibits finite deuterium and solvent deuterium isotope effects.
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Waals contacts from Ala-164 and Leu-94 (Fig. 9B). It is these interactions that are in common with α-Kg binding. However, the α-amino of lysine is within hydrogen bonding distance to Tyr-297* (Fig. 9B) and may have a role of a general acid in the catalytic mechanism. Comparing the structures of TtHCS and ScHCS, the C-terminal small domain I is similar to that of the corresponding domain of SpHCS (10, 16). Lysine binding in these structures is also similar with His-72 in TtHCS (His-103 in ScHCS) playing an important role by changing its side chain orientation to stabilize Arg-12. His-72 is conserved among lysine-sensitive HCSs, suggesting a role in feedback inhibition by lysine. In contrast, feedback inhibition by leucine in Mycobacterium tuberculosis isopropylmalylate synthase (MtIPMS) occurs by binding of the inhibitor to a separate regulatory domain linked to the C-terminal catalytic domain (28). A mechanism similar to that proposed for isopropylmalylate synthase is expected for LiCMS because it also possesses a C-terminal regulatory domain.

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