Influence of Allosteric Regulators on Individual Steps in the Reaction Catalyzed by *Mycobacterium tuberculosis* 2-Hydroxy-3-oxoadipate Synthase*

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**Background:** 2-Hydroxy-3-oxoadipate synthase is predicted to be essential for growth of *Mycobacterium tuberculosis* and is subject to allosteric regulation.

**Results:** The role of regulators was characterized by kinetics and detection of thiamin diphosphate-bound covalent intermediate distribution.

**Conclusion:** AcCoA activates steps leading to a predecarboxylation intermediate; GarA chiefly inhibits postdecarboxylation steps.

**Significance:** This novel regulatory regime in thiamin diphosphate enzymes provides new leads to inhibition.

Allosteric regulation often controls key branch points in metabolic processes. *Mycobacterium tuberculosis* 2-hydroxy-3-oxoadipate synthase (HOAS), a thiamin diphosphate (ThDP)-dependent enzyme, produces 2-hydroxy-3-oxoadipate using 2-ketoglutarate and glyoxylate. The proposed chemical mechanism in analogy with other ThDP-dependent carboligases involves multiple ThDP-bound covalent intermediates. Acetyl coenzyme A is an activator, and GarA, a forhead association domain-containing protein known to regulate glutamate metabolism, is an allosteric inhibitor of HOAS. Steady state kinetics using assays to study the first half and the full catalytic cycle suggested that the regulators act at different steps in the overall mechanism. To explore the modes of regulation and to test the effects on individual catalytic steps, we performed circular dichroism (CD) studies using a non-decarboxylatable 2-ketoglutarate analog and determined the distribution of THDP-bound covalent intermediates during the steady state of the HOAS reaction using one-dimensional 1H gradient carbon heteronuclear single quantum coherence NMR. The results suggest that acetyl coenzyme A acts as a mixed V and K type activator and predominantly affects the predecarboxylation steps. GarA does not inhibit the formation of the predecarboxylation analog and does not affect the accumulation of the postdecarboxylation covalent intermediate derived from 2-ketoglutarate; however, it decreases the abundance of the product ThDP adduct in the HOAS pathway. Thus, the two regulators act on different halves of the catalytic cycle in an unusual regulatory regime.

Thiamin diphosphate (ThDP)2-dependent 2-ketoglutarate: glyoxylate carboligase activity and the resultant production of 2-hydroxy-3-oxoadipate (HOA) reported in both bacterial and mammalian cell extracts (1–5) are assigned to the E1 component of the 2-ketoglutarate dehydrogenase complex (E1o) based on experiments with the purified enzymes in vitro (see Scheme 1). In mycobacterial cell extracts, the 2-ketoglutarate dehydrogenase complex activity could not be detected, and the E2o component could not be identified (6). However, the product of the gene Rv1248c (annotated as the E1o of *Mycobacterium tuberculosis* 2-ketoglutarate dehydrogenase complex) could catalyze the synthesis of HOA in vitro using either 2-ketoglutarate and glyoxylate or a small molecule extract of mycobacteria as a source of substrates. Overexpression of Rv1248c in *M. tuberculosis* led to an increase in the abundance of HOA (detected as 2-hydroxylevulinate by mass spectrometry), suggesting that the HOA synthase activity arises from Rv1248c in vivo (7). The metabolic fate of HOA is unknown. HOA synthase activity has been implicated in glyoxylate detoxification in mouse liver (8).

Acetyl coenzyme A (AcCoA) was recently identified as an allosteric activator, and GarA (Rv1827) was identified as an allosteric inhibitor of *Mycobacterium smegmatis* 2-ketoglutarate decarboxylase, a homolog of HOAS (9), with respect to both its 2-ketoglutarate decarboxylase activity and its HOA synthase activity. However, GarA inhibited the two activities to a different extent. To gain more insight into the function of this still enigmatic enzyme, we attempted to identify the individual chemical steps affected by these allosteric modulators. We used circular dichroism (CD) spectroscopy to monitor accumulation of a stable predecarboxylation intermediate analog and chemical quench NMR to assess rate-limiting steps to gain from *M. tuberculosis* and overexpressed in *E. coli*; HOA, 2-hydroxy-3-oxoadipate; MSP, methyl succinyl phosphate; MSP-ThDP, adduct of MSP with ThDP at the C2 position; enamine, postdecarboxylation intermediate from the predecarboxylation adduct of 2-ketoglutarate with ThDP at the C2 position; HBThDP, C2-hydroxybutanate-ThDP; HOA-ThDP, adduct of HOA with ThDP at the C2 position; E1o, E1 component of the 2-ketoglutarate dehydrogenase complex; Bis-Tris, bis(2-hydroxyethyl)aminomethyl(phosphorylmethyl) methane; SUMO, small ubiquitin-like modifier; 2KG, 2-ketoglutarate.© 2013 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
further insight into the various reactions catalyzed by HOAS under multiple conditions. These methods allowed us to study events in active sites containing ThDP so that the effect of allosteric regulation on individual catalytic steps could be determined.

**EXPERIMENTAL PROCEDURES**

**Materials—** Alcohol dehydrogenase from horse liver, β-mercaptoethanol, β-NADH, Bis-Tris, Na₂EDTA, acetyl coenzyme A, potassium phosphate, disodium 2-ketoglu-tarate, glyoxylate, MES, phenylmethanesulfonyl fluoride (PMSF), potassium ferricyanide, carbencillin, kanamycin, MgCl₂, thiamin hydrochloride, and thiamin diphosphate were obtained from Sigma. Dithiothreitol (DTT) was from USB Corp. (Cleveland, OH), and isopropyl β-d-1-thiogalactopyranoside was from Denville Scientific (Metuchen, NJ). Methyl succinyl phosphonate (MSP) disodium salt was synthesized as reported (10). The synthesis of [C₂,C₆-¹³C₂]ThDP has been reported (11).

**Immunoprecipitation, Protein Identification, and N-terminal Sequence**—Endogenous HOAS from *M. tuberculosis* lysates was immunoprecipitated as described earlier (7). The samples were subjected to SDS-PAGE (7.5%), and proteins migrating at the *M*ₐ ~135,000 band were subjected to trypic digestion followed by LC/MS analysis to detect enrichment of HOAS. In a duplicate experiment, after SDS-PAGE (7.5%) separation, the proteins were transferred to a PVDF membrane, and the HOAS-enriched band was subjected to Edman degradation for N-terminal sequence determination.

**Cloning, Overexpression, and Purification of HOAS**—The *M. tuberculosis* ketoglu-tarate decarboxylase (kdg) gene (Rv1248c) was PCR-amplified from H37Rv genomic DNA using the forward primer 5'-TCGAGGGAAACGCCATATGGCACA- CATAA-3' and reverse primer 5'-ATGTCTCTCGAGCGGTTAAGCTTAGGAA-3'. Restriction sites for Ndel and HindIII, respectively, are underlined. The PCR fragment was cloned into the pET-11c vector, and the resulting plasmid was transformed into *Escherichia coli* BL21(DE3) CodonPlus-RILP competent cells. Cultures were grown in LB medium containing 50 mg/liter carbenicillin, 1 mM thiamin, and 1 mM MgCl₂ at 37 °C with shaking at 220 rpm. At an *A₆₀₀* of ~0.6–0.8, target protein overexpression was induced with 0.5 mM isopropyl β-d-1-thiogalactopyranoside, and the culture was incubated at 18 °C while shaking at 220 rpm overnight. Harvested cells were washed with PBS and resuspended in 40 ml of 20 mM KH₂PO₄ (pH 7.4) containing 0.2 mM ThDP and 5 mM MgCl₂ (Buffer A). PMSF (1 mM final concentration), lysozyme (0.6 mg/ml final concentration), one protease inhibitor mixture tablet, and 1 μg/ml DNase were added to the cell suspension and incubated at 37 °C for 30 min. The cell suspension was passed twice through a French press operating at 1000 p.s.i. in an ice-cooled chamber. All further operations were performed at 4 °C unless otherwise noted. The lysates were clarified by centrifugation at 52,000 × g for 25 min. The supernatant was diluted to 200 ml in Buffer A, and solid (NH₄)₂SO₄ was added to make the solution 15% (w/v) in (NH₄)₂SO₄ with stirring for 30 min. The resulting suspension was centrifuged at 35,000 × g for 25 min, and solid (NH₄)₂SO₄ was added to the supernatant to make the solution 45% (w/v) saturated with stirring for 30 min. The resulting clear white suspension was centrifuged at 35,000 × g for 25 min, and the pellets were collected, dissolved in Buffer A, and dialyzed against 4 liters of Buffer A overnight. Batch purification was performed using a Q Sepharose FF column (20 ml). The column was washed with Buffer A, and bound proteins were eluted with Buffer B (Buffer A + 1 M NaCl) with a linear gradient in 15 column volumes. Fractions containing HOAS (as determined by SDS-PAGE) were pooled, concentrated, and dialyzed against Buffer A overnight. Additional purification was achieved by using a Mono Q (8-ml) column equilibrated with Buffer A and eluted using Buffer B with a linear gradient in 11 column volumes. Fractions containing HOAS were pooled, concentrated, flash frozen in liquid N₂, and stored at ~80 °C until use. In a typical batch, protein was purified to ~90% homogeneity according to Coomassie staining and densitometry analysis. The N-terminal sequence of a sample of the purified recombinant protein was confirmed by Edman degradation.

**Cloning, Overexpression, and Purification of GarA**—The *M. tuberculosis* garA gene (Rv1827) was PCR-amplified from H37Rv DNA using the forward primer 5’-TCAGTGACGGA-CATGGAACCCCGG-3’ and the reverse primer 5’-TCACGGGCCCGCGTACT-3’. The PCR fragment containing additional TCA coding for N-terminal Ser (to assist in complete Ulp protease cleavage of the SUMO tag) was cloned into the pET-SUMO vector using the TA Cloning® method. After the sequence had been verified, the resulting plasmid was transformed into *E. coli* BL21(DE3) CodonPlus-RILP competent cells. Three liters of LB containing 50 mg/liter kanamycin were inoculated with 60 ml of overnight starter culture and incubated at 37 °C with shaking at 220 rpm. At an *A₆₀₀* of ~0.6–0.8, target protein overexpression was induced with 1.0 mM isopropyl β-d-1-thiogalactopyranoside, and the culture was incubated at 37 °C while shaking at 220 rpm for 6 h. The harvested cells were washed with PBS and resuspended in 40 ml of 25 mM Tris (pH 8.0) (Buffer C). PMSF (1 mM final concentration), lysozyme (0.6 mg/ml final concentration), one protease inhibitor mixture tablet, and 1 μg/ml DNase were added to the suspension and incubated in a 37 °C water bath for 30 min. The lysozyme-treated cell suspension was passed twice through a French press operating at 1000 p.s.i. in an ice-cooled chamber. All further operations were performed at 4 °C unless otherwise noted. The lysed cells were clarified by centrifugation at 35,000 × g for 25 min. Batch purification was performed with a His-Trap FF (5 ml) column washed with Buffer C, and the protein was eluted with Buffer D (Buffer C + 500 mM imidazole) with a linear gradient in 7 column volumes. Fractions containing the target SUMO fusion protein were pooled and treated with ubiquitin-like protein 1 (Invitrogen) at 1 μg/mg protein and incubated at ambient temperature for 6 h. Next, the mixture was loaded onto a His-Trap FF column (5 ml) equilibrated with Buffer C and eluted with Buffer D. The flow-through containing GarA without the fusion SUMO tag was concentrated and dialyzed against Buffer C overnight. Aliquots of the dialyzed protein were flash frozen in liquid N₂ and stored at ~80 °C until use.
Allosteric Regulation during Individual Catalytic Steps

Enzyme Assays—Protein concentrations were determined using the Bradford assay method (12). Ferricyanide reductase assays were performed as described (6). Succinate semialdehyde production was monitored using horse liver alcohol dehydrogenase in the presence of NADH, and the disappearance of NADH was monitored at 340 nm. The activity was measured in the presence of 20 mM 2-ketoglutarate in a 1-ml volume of standard assay buffer (20 mM Bis-Tris, 1 mM ThDP, 5 mM MgCl₂, 0.2 mg/ml NADH, 0.08 mg/ml alcohol dehydrogenase (pH 6.5)) at 37 °C. The reaction was started with addition of 1 μM HOAS. Assays were performed using a Uvikon XL UV-visible spectrophotometer (SI Analytics GmbH, Mainz, Germany) equipped with a circulating water bath and thermostap- ers operating at 37 °C. Assays were performed in triplicate, and the experiment was performed at least twice.

pH Dependence of Enzyme Activity—A three-component buffer system (50 mM acetic acid, 50 mM MES, and 100 mM Tris) (13) containing additional 500 μM ThDP and 5 mM MgCl₂ was used in the pH range 4.5–8.5 to assay for the ferricyanide reductase and carboligase activities. The pH dependence of the kinetic parameters for the ferricyanide reductase activity was determined using the assay modified for the plate reader format described in the following section.

CD Spectroscopy—CD spectra were recorded on a Chirascan CD spectrometer (Applied Photophysics, Leatherhead, UK) in the presence of 20 mM 2-ketoglutarate in a 1-ml volume of a UV-transparent clear bottom plates using a Spectramax M5 plate reader (Molecular Devices, Sunnyvale, CA).

Data Fitting—Kinetic data were fitted using the nonlinear, least square, curve fitting program of SigmaPlot v.10.0 for Windows. The data points are the means of experimental replicates, and the error bars are the standard deviation. The solid lines are the regression fits. Standard errors are reported with the fitted constants.

Plate Reader Assays—A typical ferricyanide reductase assay reaction mixture (200 μl) in 20 mM Bis-Tris (pH 6.5) contained 5 mM MgCl₂, 1.6 mM K₃Fe(CN)₆, 200–1000 nM HOAS, and varying amounts of ThDP (0–500 μM), 2-ketoglutarate (0–20 mM), AcCoA (0–2000 μM), MSP (0–2000 μM), or GarA (0–30 μM). The time-dependent decrease in absorbance at 420 nm was monitored over 20 min at 37 °C. The linear region of the progress curves was used to calculate the steady state velocities. All assays were carried out in triplicate in Corning 96-well transparent clear bottom plates using a Spectramax M5 plate reader (Molecular Devices, Sunnyvale, CA). The kinetic parameters kₐₑ₅, Kₐ₉, and kₐₑ₅/Kₐ₉ obtained from Michaelis-Menten plots upon varying either 2-ketoglutarate or ThDP in the presence of various fixed concentrations of the allosteric regulators were fit to Equation 3 for hyperbolic increase, Equation 4 for hyperbolic increase with reversal at high concentrations, or Equation 5 for hyperbolic decrease.

\[
\beta = \beta^0 + \beta_{\text{max}} \times \frac{[L]}{K_L + [L]} \\
\beta = \beta^0 + \frac{\beta_{\text{max}} 	imes [L]}{K_L + [L]} \times \left(1 + \frac{[L]}{K_R}ight) \\
\beta = \beta^0 - \beta_{\text{min}} \times \frac{[L]^n}{K_L + [L]^n}
\]

In these expressions, L is the concentration of the varied regulator, \( \beta \) is the value of the kinetic parameters at various L, \( \beta^0 \) is the value in the absence of the regulator, \( \beta_{\text{max}} \) is the maximal value, and \( \beta_{\text{min}} \) is the minimal value. \( K_L \) is the concentration of the regulator at which the half-maximal regulatory effect is observed on each of the parameters. \( K_R \) is the constant for loss of activation, and n is the Hill coefficient.

\( K_L \) of MSP was estimated by global fit of inhibition performed using Equation 6 where the varied substrate S is 2-ketoglutarate.

\[
(\text{v}/E) = \frac{(V_{\text{max}}/E) \times S}{S + K_m(1 + [\text{MSP}]/K_L)}
\]

The effect of regulators on the steady state linear velocity in the (R)-HOA synthase assay was determined by fitting the data to Equation 3 for AcCoA and Equation 5 for GarA. Here, \( \beta \) is the steady state linear velocity v/E where E is the enzyme concentration. L is the concentration of the regulator, and \( K_L \) is the apparent activation or inactivation constant.

Data for GarA, plotted in the fractional inhibition form, were fitted to Equation 3. Here, \( \beta \) is the percentage of inhibition, \( \beta^0 \) was set to zero, and \( \beta_{\text{max}} \) is the maximal percentage of inhibition. L is the concentration of GarA, and \( K_L \) is the apparent inactivation constant, \( K_{\text{inact}} \).
$K_i$ of GarA was estimated by global fit of inhibition performed using Equation 7 where the varied substrate $S$ is glyoxylate. All data were fit by nonlinear regression using SigmaPlot v.10.0.

$$
\frac{v}{E} = \frac{(V_{\text{max}}/E) \times S}{(S + K_m) \times (1 + [\text{GarA}]/K)}
$$

\text{(Eq. 7)}

**Titration of HOAS with MSP**—To a 2.4-ml cuvette containing HOAS (2.75 mg/ml; 20 $\mu$M) in 20 mM KH$_2$PO$_4$ (pH 7.0) with additional 200 $\mu$M ThDP and 5 mM MgCl$_2$, incremental amounts of MSP were added from a 1 M stock solution to obtain the desired final concentrations (0–20 mM). After mixing for 10 s and incubation for an additional 30 s, near-UV (280–400 nm) CD spectra were collected at 25 °C. The procedure was essentially the same for experiments with HOAS (20 $\mu$M) in the presence of saturating concentrations of the allosteric regulator, either AccCoA (500 $\mu$M) or GarA (50 $\mu$M). For quantitative analysis, the amplitude of the CD band pertaining to the 1',4'-iminopyrimidine tautomer of the MSP-ThDP complex at 302 nm corrected for dilution was plotted against MSP concentration. Apparent dissociation constants ($K_{app}$) were calculated by fitting the data to the Hill function described in Equation 8 using SigmaPlot v.10.0.

$$
\text{CD}_{302} = \text{CD}_0 + \frac{\text{CD}_{302}^{\text{max}} \times [\text{MSP}]^n}{(K_{app}^{\text{ppp}} + [\text{MSP}]^p)}
$$

\text{(Eq. 8)}

In this expression, $\text{CD}_{302}$ is the observed CD signal at the given wavelength, $\text{CD}_0$ is the CD signal of the protein at this wavelength in the absence of MSP, $\text{CD}_{302}^{\text{max}}$ is the maximum CD signal at saturation with MSP, $[\text{MSP}]^n$ is the concentration of substrate analog, and $n$ is the Hill coefficient.

**Steady State Distribution of ThDP-bound Covalent Intermediates**—Apo-HOAS (25 mg/ml; 185.2 $\mu$M) was incubated with 2 eq of [C$_2$C$_6$-$^{13}$C] ThDP (370.4 $\mu$M) in 20 mM KH$_2$PO$_4$ buffer (pH 7.0) containing MgCl$_2$ (5 mM) at 4 °C for 30 min. For experiments with the allosteric modulators, the enzyme reconstituted with labeled ThDP was divided into 200-$\mu$l aliquots, 2 eq of GarA (370.4 $\mu$M) or AccCoA (370.4 $\mu$M) were added, and the mixture was incubated for 30 min on ice. Typical reactions were started by addition of substrates to a final concentration of 40 mM each to 200 $\mu$l of the enzyme aliquot at 25 °C, and the reaction was stopped after 30 s by addition of 100 $\mu$l of 12.5% TCA in 1 M DCI/D$_2$O. The carboligase reaction in the presence of activator AccCoA was stopped at 5 s. The quenched mixtures were centrifuged at 15,700 $\times$ g for 30 min, and the supernatant was filtered using a Gelman nylon Acrodisc (0.45 $\mu$m). D$_2$O (80 $\mu$l) was added to the filtered samples. The resultant samples were analyzed by a one-dimensional $^1$H gradient carbon heteronuclear single quantum coherence experiment on a Varian 600-MHz NMR instrument. Data acquisition was at 25 °C, and sample pH was ~0.75. 2816 transients in the $^1$H dimension were collected with a recycle delay time of 2 s. Data processing (exponential window function and zero filling) and analyses (base-line correction, integration, and signal-to-noise ratio estimates) were performed using ACD/NMR processor academic edition (v12.01, Advanced Chemistry Development, Inc., Toronto, Canada). The relative integrals of C6'-H signals of respective ThDP-bound covalent intermediates represent their relative ratios after correction for the amount of unbound ThDP estimated using Equations 9 and 10.
Allosteric Regulation during Individual Catalytic Steps

Here, \([E - \text{ThDP}]\) represents the concentration of HOAS-ThDP complex, \([\text{ThDP}]_o\) and \([\text{ThDP}]_f\) represent concentrations of free and total ThDP, and \(K_d\) is the dissociation constant.

RESULTS

Cloning, Expression, and Purification

The bioinformatics prediction of the N terminus of HOAS has been revised since the \(M.\) \(tuberculosis\) enzyme was first studied (14). To ensure that our recombinant protein included the native N terminus, we identified the N-terminal start sequence of endogenous HOAS. We immunoprecipitated HOAS from \(M.\) \(tuberculosis\) H37Rv lysates using \(\alpha\)-HOAS antibodies (7) and subjected the band corresponding to the target protein to Edman sequencing. The first 5 amino acids were determined to be ANISS in agreement with recent observations (15). Next, HOAS was cloned into the pET-11c vector, and tagless recombinant HOAS was overexpressed and purified to \( \geq 90\% \) purity as judged by SDS-PAGE. The N-terminal start sequence of the recombinant enzyme was confirmed by Edman sequencing. GarA was PCR-amplified and cloned into the pET-SUMO vector, and recombinant GarA was overexpressed and purified to \( \geq 95\% \) purity. Approximately 15 mg of pure HOAS and 15 mg of pure GarA were obtained per liter of culture.
Detection and Characterization of HOA

Carboligase products of ThDP-dependent enzymes can be detected by CD spectroscopy in the near-UV region (250–400 nm) because the products are chiral. A negative band at \( \lambda_{\text{max}} = 278 \text{ nm} \) showed a time-dependent increase in the presence of HOAS (500 nM), 2-ketoglutarate, and glyoxylate (Fig. 1A). This band, suggestive of accumulation of a chiral product, was observed in the supernatant after enzyme removal and did not appear when one of the three components was omitted. Earlier, \(^1\)H NMR and LC-MS analyses of this reaction assigned the product as 2-hydroxy-3-oxoadipate, which is decarboxylated non-enzymatically to hydroxylevulinate (7). By analogy with carboligase reactions of other ThDP-dependent enzymes (16, 17), we assigned the observed band to the \((R)\)-HOA enantiomer. The progress curves measured at 278 nm allowed determination of the steady state linear velocities of HOAS-catalyzed production of \((R)\)-HOA (Fig. 1B) (17). At longer times, the negative CD band decreased, consistent with earlier observations of decarboxylation of \((R)\)-HOA to achiral 2-hydroxylevulinate and similar to the decarboxylation of acetalactate to acetoin or of tartronate semialdehyde to glycolaldehyde (17). Fitting this latter curve to a first-order equation yielded a half-life of 28.9 min for \((R)\)-HOA with a first-order rate constant for decarboxylation of \((4 \pm 0.05) \times 10^{-4} \text{ s}^{-1}\), which is within the error range of the measured steady state linear velocities (Fig. 1C).

Steady State Kinetic Studies of (R)-HOA Synthase (HOAS) Activity

**pH Dependence of Activity**—The pH dependence of 2-ketoglutarate-dependent ferricyanide reductase activity (assay for formation of the postdecarboxylation intermediate; Scheme 1) and HOAS activity showed similar bell-shaped profiles in the range of 4.5–8.5 with maxima at \( \sim 6.5 \), suggesting that both reactions proceed through a common pH-dependent rate-limiting step (Fig. 2A).

**Steady State Kinetic Parameters**—Substrate dependence of the HOAS reaction in the presence of a 10 mM concentration of the fixed substrate and a 0–50 mM concentration of the varied substrate yielded hyperbolic Michaelis-Menten plots for 2-ketoglutarate, and saturation plots with glyoxylate showed linear substrate inhibition at high concentrations (Fig. 2, B and C). The kinetic parameters obtained after fitting the data to Equations 1 and 2 are summarized in Table 1. Because of substrate inhibition at high concentrations, Lineweaver-Burk plots obtained by varying the concentration of 2-ketoglutarate at fixed variable concentrations of glyoxylate were nonlinear (Fig. 3, A and B), and global fitting of data to deduce the kinetic mechanism and the order of substrate addition was not performed. Linear regions in Fig. 3, A and B, were used to obtain secondary plots (Fig. 3, C and D). In the secondary plot in Fig. 3C, the intercept drops, reaching saturation, whereas the slope increases linearly with glyoxylate. This is evocative of a ping-pong kinetic mechanism where glyoxylate displays competitive substrate inhibition with respect to 2-ketoglutarate as described by Cleland (18).

Allosteric Regulation during Individual Catalytic Steps

AccoA was shown to be a potent activator of \(M. \text{smegmatis}\) ketoglutarate decarboxylase that binds at a shallow pocket on the surface of the enzyme some 40 Å away from the active site (9). Also, GarA was shown to inhibit HOAS and glutamate dehydrogenase allosterically and to activate glutamine synthetase, thus acting as a general regulator of glutamate metabolism in mycobacteria (19). We tested the effect of these allosteric regulators on steps leading to (i) addition of ThDP to 2-ketoglutarate and...
decarboxylation by the ferricyanide reductase assay and (ii) (R)-HOA synthesis using the CD assay.

Analyses of the steady state parameters from the ferricyanide reductase assay revealed that AcCoA increases the $k_{\text{cat}}$ by over 3-fold and decreases the $K_m$ for 2KG by 6-fold, suggesting that it acts as a mixed V and K type activator to produce a 20-fold enhancement in $k_{\text{cat}}/K_m$. With respect to ThDP, both $k_{\text{cat}}$ and $K_m$ for ThDP increased and $k_{\text{cat}}/K_m$ for ThDP decreased with increasing AcCoA. Within the range tested, $k_{\text{cat}}/K_m$ for ThDP was 13.9 ± 3.3 with a 17-fold rate enhancement (Fig. 6B). In addition, the inhibition by GarA was non-competitive with respect to glyoxylate (Fig. 6C). Although the $K_{\text{inact}}$ for GarA was similar from both assays, the different rate reductions suggested that GarA possibly affected the second half of the catalytic cycle. We hypothesized that this could be due to the differential role of the allosteric regulators at the individual catalytic steps. To test this hypothesis, we used recently developed CD and NMR methods (21).

### Allosteric Regulation during Individual Catalytic Steps

Substrate Analog as a Mechanism-based Inhibitor—Phosphonate analogs of 2-ketoacids were shown earlier to be potent mechanism-based inhibitors of ThDP-dependent decarboxylases (22, 23). They form a covalent ThDP adduct in a mechanism similar to the natural substrates and accumulate as non-decarboxylatable analogs of the predecarboxylation intermediate (Fig. 7E). The enzyme-bound form of this intermediate is also exclusively detected by near-UV (280–400 nm) CD spectroscopy due to the induced chirality of active site-bound ThDP (V conformation) and stabilization of the $1',4'$-iminopyrimidine chromophore during the catalytic cycle (24).

### Table 1

| Fixed substrate$^a$ | Variable substrate | $k_{\text{cat}}$ $s^{-1}$ | $K_m$ mm | $K_i$ mm | $k_{\text{cat}}/K_m$ m$^{-1}$ s$^{-1}$ |
|---------------------|--------------------|---------------------------|---------|---------|---------------------------------|
| Glyoxylate          | 2-Ketoglutarate    | 4.25 ± 0.13               | 0.84 ± 0.1 | 3.2 ± 0.76 | 53.1 ± 13.9$^b$ |
| 2-Ketoglutarate     | Glyoxylate         | 5.29 ± 0.47               | 3.2 ± 0.76 | 3.2 ± 0.76 | 1653                            |

$^a$ The fixed substrates were held at 10 mM.

$^b$ Substrate inhibition constant at high concentration of glyoxylate.
We tested MSP using the ferricyanide reductase assay in the absence and presence of AcCoA. The mechanism of inhibition was competitive with respect to 2-ketoglutarate under the different conditions tested (Fig. 7, A and B, and Table 3). The calculated $K_i$ values were $K_i^{\text{calc}} = 527.4 \, \mu M$ for 0 $\mu M$ AcCoA, $K_i^{\text{calc}} = 439.3 \, \mu M$ for 50 $\mu M$ AcCoA, and $K_i^{\text{calc}} = 143.2 \, \mu M$ for 500 $\mu M$ AcCoA. These values are similar to the $K_i$ for 2-ketoglutarate under these conditions, suggesting that MSP mimics 2-ketoglutarate on the activated enzymes.

Effect of Allosteric Regulators on Accumulation of Predecarboxylation Intermediates—Upon titration of HOAS (20 $\mu M$) with MSP (0–20 mM), a positive band centered at 302 nm developed and displayed saturation at higher concentrations (Fig. 7C). The spectra were reminiscent of analogous experiments with phosphonate analogs (10, 24), and the positive CD band observed at 302 nm could be assigned to the $1$, $4$-iminopyrimidine tautomer of the MSP-ThDP adduct. The titration data fit to Equation 2 revealed hyperbolic binding characteristics ($n = 1.0$) with $K_i^{\text{calc}} = 575 \pm 38 \, \mu M$, similar to the $K_i^{2KG}$ of 570 $\pm 80 \, \mu M$ for 2-ketoglutarate (Fig. 7D). In the presence of saturating AcCoA, the $K_i^{\text{calc}} = 158 \pm 10 \, \mu M$ was again similar to the $K_i^{2KG}$ of...
169 ± 22 µM in the ferricyanide reductase assay (Table 3), suggesting that the activation observed in the presence of AcCoA is achieved by improving the affinity for the substrate or by stabilizing the incipient lactyl-type intermediate (Fig. 7D). In the presence of saturating GarA (50 µM), a positive CD band at 302 nm saturating at a maximal amplitude similar to the GarA free enzyme was again observed, suggesting that the binding of GarA does not inhibit the formation of MSP-ThDP, the predecarboxylation adduct. However, the increased $K_d$ of 2300 ± 235 µM suggests that the affinity for the substrate is weaker compared with the unregulated enzyme (Fig. 7D), consistent with our observations with the ferricyanide reductase assay (Table 3).

### Detection of ThDP-bound Covalent Intermediates during Steady State of Various Reactions

To determine the effect of the presence of allosteric regulators on the first half and full catalytic cycle of the HOAS reaction, steady state accumulation of ThDP-bound covalent intermediates was estimated using HOAS reconstituted with [C2,C6-13C2]ThDP. The assignment of NMR resonances to the covalent ThDP intermediates was based on the following observations. 1) The E. coli ketoglutarate dehydrogenase complex E1o component accepts succinate semialdehyde in the reverse direction, and the adduct C2-hydroxybutanoate-ThDP could be observed at 7.37 ppm (data not shown). The 2-ketoglutarate-ThDP adduct could not be detected in the forward direction, so the C6'-H chemical shift is not available for this intermediate. The assignment of C6'-H chemical shifts for glyoxylate-ThDP adducts was achieved by Kaplun et al. (25). Finally, we assigned the new resonance at 7.41 ppm to the HOA-ThDP adduct (product-ThDP adduct) based on 1) the product formed, 2) the expected similarity of the chemical mechanism to other ThDP carboligases, and 3) the comparison with the known trend of C6'-H chemical shift of various other C2-substituted ThDP intermediates.

*M. tuberculosis* HOAS-catalyzed conversion of 2-ketoglutarate to succinate semialdehyde involves slow (0.02–0.05 s⁻¹) than the rate of oxidation of the enamine in the $K_{3Fe(CN)6}$ assay (∼1.0 s⁻¹). In the presence of 2-ketoglutarate, the 2-ketoglutarate-ThDP adduct was not detected, whereas C2-hydroxybutanoate-ThDP (HBThDP) occupied 35.2% of the active sites, and ThDP occupied 64.8% of the active sites (Fig. 8A and Table 4). Taken together, these observations suggest that 1) the decarboxylation step is much faster than the addition of 2-ketoglutarate to ThDP, and 2) succinate semialdehyde release is the rate-limiting step for the first half of the catalytic cycle (Fig. 8, *reaction scheme*). In the presence of AcCoA, again the 2-ketoglutarate-ThDP adduct was not detected, and HBThDP and ThDP were detected at 39.3 and 60.6%, respectively (Fig. 8B). Similar results (HBThDP, 34.5%; ThDP, 65.5%) were observed in the presence of saturating GarA, suggesting that GarA does not significantly affect the decarboxylation step and the steady state concentration of HBThDP (Fig. 8C).

In the presence of both 2-ketoglutarate and glyoxolate, the 2-ketoglutarate-ThDP adduct was not detected, whereas HBThDP, HOA-ThDP adduct, and ThDP were detected at 33.1, 9.6, and 57.3%, respectively (Fig. 8D). No resonances pertaining to the glyoxylate-ThDP adducts were observed. This suggests that the addition of 2-ketoglutarate to ThDP, addition of glyoxylate to the enzyme, and release of HOA are the slow steps compared with the decarboxylation of 2-ketoglutarate-ThDP adduct (Fig. 8, *reaction scheme*). In the presence of saturating AcCoA, the fractional occupancies were 41.8, 11.3, and 46.9% for HBThDP, HOA-THDP, and ThDP, respectively (Fig. 8E). In the presence of saturating GarA, the covalent intermediates HBThDP (25%) and HOA-ThDP (3.75%) occupied a lower fraction of the active sites compared with the earlier cases (Fig. 8F).

### DISCUSSION

There is complexity to the mechanism of this enzyme as it could result in one of three outcomes: 1) formation of succinate semialdehyde via 2-ketoglutarate decarboxylation, a non-oxidative decarboxylase pathway; 2) formation of HOA, a carboxylase activity; and 3) formation of a putative precursor to reductive succinylation of a putative E2 component in a manner expected in most prokaryotic and eukaryotic cells for 2-oxoacid dehydrogenase complexes. In view of results so far accumulated (6, 7, 26), it appears that pathways 1 and 2 are predominant. All three pathways are derived from ThDP-catalyzed decarboxylation of 2-ketoglutarate and the reaction of the enamine intermediate with either a H⁺, glyoxylate, or lipoamide bound to a putative E2, respectively.

Slow aldehyde release (pathway 1) is a familiar outcome in thiamin-dependent 2-oxoacid dehydrogenases and carboligases in the absence of acceptor substrates. Our earlier work had shown that the gene product of Rv1248c does not catalyze pathway 3. Based on work with *M. smegmatis* ketoglutarate decarboxylase, it was suggested that our use of recombinant enzymes based on a misannotated sequence and the absence of AcCoA could explain our observations (9). To test these ideas, we cloned tagless HOAS based on immunoprecipitation from *M. tuberculosis* lysates and N-terminal sequencing. We were...
Allosteric Regulation during Individual Catalytic Steps

A

\[ K_{\text{act}}^{\text{AcCoA}} = 9.92 \pm 0.44 \text{ M} \]

B

\[ K_{\text{act}} = 2.88 \pm 0.36 \text{ M} \]

C

FIGURE 6. Effect of allosteric regulators on (R)-HOA synthase reaction. A, activation by AcCoA. B, inactivation by GarA. Inset, the data plotted as percentage of inactivation. Production of (R)-HOA was monitored directly by the CD assay at 37°C in 20 mM Bis-Tris (pH 6.5) containing additional 200 μM ThDP and 5 mM MgCl₂. The concentrations of 2-ketoglutarate and glyoxylate were maintained at 10 mM, and AcCoA (0–500 μM) and GarA (0–100 μM) were varied. The data were fit to Equations 3 and 5, respectively, and black traces are the regression fit. C, mechanism of inhibition of GarA with respect to glyoxylate. Error bars represent S.D.

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showed lower turnover numbers with M. smegmatis ketoglutarate decarboxylase in earlier work by Wagner et al. (9) \( k_{\text{cat}} \sim 0.1 \text{ s}^{-1} \) for the AcCoA-activated enzyme) compared with our assays. As this study deals with the influence of allosteric regulators on HOAS catalysis, we chose pathway 2 for a detailed steady state kinetic characterization. However, the detection of intermediates and the role of allosteric regulators as elucidated here would bear significance for any of the pathways considered.

No chemical models and \(^1\)H NMR chemical shifts for the ThDP covalent intermediates in the catalytic cycle of 2-ketoglutarate-dependent enzymes are available to date. Hence, we used \([C_2,C_6]ThDP\)-reconstituted HOAS for unambiguous assignment of ThDP-derived covalent intermediates using one-dimensional \(^1\)H gradient carbon heteronuclear single quantum coherence NMR, a method that selectively detects \(^1\)H attached to \(^13\)C atoms. In the various experiments, we did not detect resonances related to buffer, substrate, or other low level contaminants; hence, new resonances can be assigned with confidence to ThDP covalent intermediates. However, a full NMR characterization of the HOA-ThDP adduct was not possible given the unstable nature of HOA \( t_{1/2} \sim 29 \text{ min} \), the anticipated starting material for the chemical synthesis of HOA-ThDP. We did not undertake NMR investigations of the ferricyanide reductase pathway because the use of \( K_{3}\text{Fe(CN)}_6 \) is incompatible with our NMR methods.

The (R)-HOA detected by our CD assay was assumed to be enantiopure for calculation of linear velocities. We were unable to trap and purify the unstable (R)-HOA to determine the true enantiomeric excess by chiral gas chromatography-coupled mass spectrometry. Thus, the \( k_{\text{cat}} \) values reported here are the lower limits. The \( k_{\text{cat}} \) values of the unactivated \( (4 \text{ s}^{-1}) \) and the AcCoA-activated enzyme \( (21 \text{ s}^{-1}) \) are comparable with those of other carboligases that use 2-ketoacid substrates, such as glyoxylate carboligase and isozymes of acetohydroxyacid synthase from various bacteria \((17, 25, 27–30)\). The rates on the unactivated enzyme are similar to those of M. tuberculosis acetohydroxyacid synthase, whereas the activated rates are comparable with those of E. coli enzymes. Variants of 2-ketoacid decarboxylases can be engineered to enhance rates of production of carboligase products. Examples include the E636A E1p variant of E. coli pyruvate decarboxylase \( (k_{\text{cat}} = 1 \text{ s}^{-1}) \) and the D28A variant of Saccharomyces cerevisiae pyruvate decarboxylase \( (k_{\text{cat}} = 0.05 \text{ s}^{-1}) \), which behave like acetolactate synthase \((31)\), and the E473Q variant of Zymomonas mobilis pyruvate decarboxylase, which catalyzes the enantioselective conversion of pyruvate and benzaldehyde to (R)-phenylacetylcarbinol at \( 2.5 \text{ s}^{-1} \) \((32)\). This comparison suggests that HOAS is indeed catalytically competent toward C–C bond-forming reactions and even surpasses the engineered variants when AcCoA activation is taken into account. Although (R)-HOA production has been identified in other species, kinetic characterization of the other enzymes \((6, 26)\) will be necessary to determine whether the M. tuberculosis enzyme exemplifies a general feature of the E1o components or whether it has diverged to become a particularly competent HOA synthase in connection with a variant tricarboxylic acid cycle in M. tuberculosis.
Allosteric Regulation during Individual Catalytic Steps

FIGURE 7. Effect of allosteric regulators on the predecarboxylation steps. Competitive inhibition by MSP, an analog of 2-ketoglutarate, in the presence of 0 μM AcCoA (A) and 50 μM AcCoA (B) is shown. The ferricyanide reductase assay was used as described under “Experimental Procedures,” and 2-ketoglutarate concentration was varied from 0.05 to 20 mM. MSP concentrations were as indicated.

C, representative near-UV (280 – 400 nm) CD spectra from the titration of 20 μM HOAS with MSP (0 – 20 mM) in 20 mM KH2PO4 at 25 °C as described in detail under “Experimental Procedures.” D, the CD amplitude of the 1',4'-iminopyrimidine form of ThDP at 302 nm was plotted against concentration of MSP. The data points were fitted to Equation 8, and the regression fit line is displayed. The effect of allosteric modulators on the Kd was determined in the presence of AcCoA (500 μM) and GarA (50 μM). E, minimal mechanism of formation of the predecarboxylation intermediate and its analog. Error bars represent S.D. mdeg, millidegrees; APH*, N1'-protonated 4'-aminopyrimidinium form of ThDP; IP, 1',4'-iminopyrimidine form of ThDP.

TABLE 3
Inhibition constants for GarA and MSP under various conditions
ND, not determined; NA, not applicable.

| Experiment conditions | Varied substrate | Inhibition type | K<sub>i</sub><sup>a</sup> | K<sub>d</sub><sup>b</sup> | Km<sup>c</sup> |
|-----------------------|-----------------|----------------|----------------|----------------|-------------|
| MSP (0 μM AcCoA)      | 2-Ketoglutarate  | Competitive    | 527.4          | 575 ± 38       | 570 ± 80    |
| MSP (50 μM AcCoA)     | 2-Ketoglutarate  | Competitive    | 439.3          | ND             | 414 ± 34    |
| MSP (500 μM AcCoA)    | 2-Ketoglutarate  | Competitive    | 143.2          | 158 ± 10       | 169 ± 22    |
| GarA in K<sub>f</sub>Fe(CN)<sub>6</sub> assay | 2-Ketoglutarate | Non-competitive | NA             | NA            | 2300 ± 235  |
| GarA in (R)-HOA synthase assay | Glyoxylate | Non-competitive | 1.36 | NA | 1846 ± 132 |

<sup>a</sup> K<sub>i</sub> values from K<sub>f</sub>Fe(CN)<sub>6</sub> assay.
<sup>b</sup> K<sub>d</sub> for MSP-ThDP from CD titration compared with K<sub>i</sub> (in last column) for 2-ketoglutarate in the presence of various concentrations of AcCoA or GarA in K<sub>f</sub>Fe(CN)<sub>6</sub> assay.
FIGURE 8. Distribution of ThDP-bound intermediates during steady state of HOAS reaction. The C6-^1^H fingerprint regions in one-dimensional ^1^H gradient carbon heteronuclear single quantum coherence NMR spectra acquired with supernatant after acid quench of the reaction of HOAS (25 mg/ml) with 2-ketoglutarate (40 mM) (A), HOAS (25 mg/ml) with 2-ketoglutarate (40 mM) in the presence of AcCoA (2 eq) (B), HOAS (25 mg/ml) with 2-ketoglutarate (40 mM) in the presence of GarA (2 eq) (C), and HOAS (25 mg/ml) with 2-ketoglutarate (40 mM) and glyoxylate (40 mM) (D), HOAS (25 mg/ml) with 2-ketoglutarate (40 mM) and glyoxylate (40 mM) in the presence of AcCoA (2 eq) (E), and HOAS (25 mg/ml) with 2-ketoglutarate (40 mM) and glyoxylate (40 mM) in the presence of GarA (2 eq) (F) are shown. All reactions were at 25 °C in the presence of 2 eq of [C2,C6/H11032-13C2]ThDP to serve as internal standard. Reaction E was stopped at 5 s; all others were stopped at 30 s. All spectra were acquired at 25 °C and pH 0.75. Acquisition parameters are described under “Experimental Procedures.” Numbers in red are the relative integral values, and numbers in blue are the 13C6/^1^H chemical shifts. The HOAS reaction scheme is shown at the bottom.
**TABLE 4**

Steady state distribution of covalent ThDP intermediates under various conditions

| Reaction conditions | ThDP   | 2KG-ThDP | HBThDP | HOA-ThDP |
|---------------------|--------|----------|--------|----------|
| A) 2-Ketoglutarate   | 64.8 ± 0.3 | ND       | 35.2 ± 0.9 | NA       |
| B) 2-Ketoglutarate + AcCoA | 60.7 ± 0.2 | ND       | 39.3 ± 0.6 | NA       |
| C) 2-Ketoglutarate + GarA | 65.5 ± 0.3 | 34.5 ± 1.1 | NA       |
| D) 2-Ketoglutarate + glyoxylate | 57.3 ± 0.6 | 33.1 ± 1.4 | 9.6 ± 1.2 |
| E) 2-Ketoglutarate + glyoxylate + AcCoA | 46.9 ± 0.4 | 41.8 ± 1.0 | 11.3 ± 1.1 |
| F) 2-Ketoglutarate + glyoxylate + GarA | 71.2 ± 0.3 | 25.0 ± 1.2 | 3.8 ± 1.1 |

*2 eq of [C2,C6-13C2]ThDP were used to ensure saturation and serve as internal standard. Percent occupancies of active sites were determined from relative integrals in Fig. 8 after correction for excess unbound ThDP and estimation of unbound enzyme using Equations 9 and 10. 

* Predecarboxylation adduct of 2-ketoglutarate with ThDP at the C2 position (2KG-ThDP) was not detected (ND).

The structure of (R)-HOA as determined by NMR and LC-MS (5, 7) and structures of various carboligase products of *E. coli* E1o, HOAS, and 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase (MenD) provide strong footing for the suggested chemical mechanism of HOAS (Scheme 1) (33), similar to other ThDP-dependent carboligases. In addition, 2-ketoglutarate-ThDP adducts could be detected, whereas glyoxylate-ThDP adducts could not be detected by NMR under the various conditions tested. Although a ping-pong kinetic mechanism can be anticipated based on the substrate addition steps being separated by an irreversible decarboxylation step, the Lineweaver-Burk plots suggest a more complex kinetic mechanism. Analysis of secondary plots for 2-ketoglutarate suggests a competitive substrate inhibition by glyoxylate as described by Cleland (18). The hysteretic lag phase in the progress curves that is indicative of the switching of the dimeric enzyme from a lower activity state to a higher activity state in the presence of 2-ketoglutarate as seen in the x-ray structures of *M. smegmatis* ketoglutarate decarboxylase (9) could contribute to the complexity of the Lineweaver-Burk plots. Pre-steady state and steady state kinetic experiments with acetohydroxacyclic synthase I mutants and deoxy-D-xylulose-5-phosphate synthase indicate that these enzymes utilize a ThDP-substrate-acceptor ternary complex where the substrate is covalently bound to ThDP, and the presence of the acceptor enhances decarboxylation rates (34–36). In sum, these examples show that ThDP enzymes can perform similar chemical steps through different kinetic mechanisms.

*M. tuberculosis* HOAS, its homolog *M. smegmatis* ketoglutarate decarboxylase, and the SucA components from other actinomycetes share sequence similarity and domain organization. OdhA from *Corynebacterium glutamicum* is similarly regulated by Odhl (GarA homolog), but its allosteric activation by AcCoA is not yet established (37, 38). ThDP-dependent decarboxylases are subject to homotropic allosteric regulation by their 2-ketoacid substrates, and in *M. tuberculosis*, a ketoacid decarboxylase involved in amino acid metabolism via the Ehrlich pathway is regulated by amino acids (39). Mammalian αβ2 heterotetrameric E1 components of pyruvate dehydrogenase are regulated by kinases that phosphorylate a residue in the active site loop, leading to inhibition of the postdecarboxylation steps. However, this does not interfere with the predcarboxylation steps (40).

In contrast, the mycobacterial enzymes are unique among the ThDP superfamily of enzymes as they are subject to two distinct modes of allosteric regulation by different regulators. The inhibitory activity of GarA is regulated by phosphorylation; binding of unphosphorylated GarA regulates HOAS. The phosphorylation state of GarA is dependent on the presence of PknB and PknG, whose expression is regulated by external stresses (19, 41, 42).

AcCoA increases the *k*\textsubscript{cat} in predecarboxylation and (R)-HOA synthase steps accompanied by a reduction in *K*\textsubscript{m} for 2-ketoglutarate and a 22-fold increase in *k*\textsubscript{cat}/*K*\textsubscript{m} for the predcarboxylation steps. In contrast, GarA produces a 2.2-fold reduction in *k*\textsubscript{cat}, a 2.4-fold reduction in *K*\textsubscript{m}, and a 4-fold reduction in *k*\textsubscript{cat}/*K*\textsubscript{m} in the predcarboxylation steps. However, a more substantial 17-fold reduction in *k*\textsubscript{cat} is observed in the (R)-HOA synthase assay, suggesting that the major ~8-fold impact on *k*\textsubscript{cat} reduction by GarA occurred on postdecarboxylation steps.

The development of modern techniques to interrogate ThDP catalysis during individual catalytic steps provided a unique opportunity to test and understand the role of various allosteric regulatory mechanisms at the individual chemical steps. First, from CD studies using substrate analogs, it is evident that AcCoA activation is pronounced in the first half of the catalytic cycle, whereas strikingly GarA does not inhibit formation of the predcarboxylation intermediate, although the substrate affinity is decreased. Second, from NMR experiments, the accumulation of the postdecarboxylation intermediate is evident in the presence of GarA, suggesting that GarA does not inhibit decarboxylation either. There is a measurable effect only on the covalent intermediate levels in the second half of the catalytic cycle (addition of glyoxylate to the enamine) in direct correlation with loss of activity in steady state kinetics.

Taken together, these results suggest that although the two regulators influence *K*\textsubscript{m} for 2-ketoglutarate they exert their *maximal* influence on different covalent bond formation steps in the catalytic cycle. AcCoA activates the predcarboxylation steps, and GarA inhibits addition of the second substrate to the central enamine intermediate. Non-competitive inhibition by GarA with respect to glyoxylate suggests that binding of GarA at a distal region elicits changes that interfere with glyoxylate binding.

*M. tuberculosis* HOAS expression is predicted to be essential for the optimal growth of *M. tuberculosis* (43). Thus, *M. tuberculosis* HOAS could be a potential target for antituberculosis drugs. Enzymes of the central carbon metabolism are difficult targets for drug development because of the evolutionarily conserved nature of the function and of the structures of the enzymes that perform them. However, even among enzymes...
that perform conserved chemical transformations by the same chemical mechanisms, the electrostatic stabilization of the transition state could differ. In addition, the presence of allosteric activators could change the nature of the transition state as in AMP nucleosidase (44, 45). Based on the dependence of $K_d$ of MSP and $K_d$ of MSP-ThDP adduct on the presence of AcCoA, we suggest that accounting for allosteric regulators could aid in developing mechanism-based inhibitors with improved potency and enhanced specificity against homologs that use different mechanisms of allostery. In addition, our findings might be of interest for studies of the regulation of other ThDP-dependent enzymes involved in the metabolism of glucose and glutamate.

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REFERENCES

1. O’Fallon, J. V., and Brosemer, R. W. (1977) Cellular localization of $\alpha$-ketoglutarate:glyoxylate carboligase in rat tissues. Biochim. Biophys. Acta 499, 321–328
2. Yamasaki, H., and Moriyama, T. (1971) Purification, general properties and two other catalytic activities of $\alpha$-ketoglutarate:glyoxylate carboligase of Mycobacterium phlei. Biochim. Biophys. Acta 242, 637–647
3. Yamasaki, H., and Moriyama, T. (1970) $\alpha$-Ketoglutarate:glyoxylate carboligase activity in Escherichia coli. Biochim. Biophys. Res. Commun. 39, 790–795
4. Yamasaki, H., and Moriyama, T. (1970) Inhibitory effect of $\alpha$-ketoglutarate:glyoxylate carboligase activity on porphyrin synthesis in mycobacterium phlei. Biochim. Biophys. Res. Commun. 38, 638–643
5. Schlossberg, M. A., Richert, D. A., Bloom, R. J., and Westerfeld, W. W. (1968) Isolation and identification of 5-hydroxy-4-kovaleric acid as a product of $\alpha$-ketoglutarate:glyoxylate carboligase. Biochemistry 7, 333–337
6. Tian, J., Bryk, R., Itoh, M., Suematsu, M., and Nathan, C. (2005) Variant tricarboxylic acid cycle in Mycobacterium tuberculosis: identification of $\alpha$-ketoglutarate decarboxylase. Proc. Natl. Acad. Sci. U.S.A. 102, 10670–10675
7. de Carvalho, L. P., Zhao, H., Dickinson, C. E., Arango, N. M., Lima, C. D., Fischer, S. M., Ouertelli, O., Nathan, C., and Rhee, K. Y. (2010) Activity-based metabolic profiling of enzymatic function: identification of $\text{Rv1248c}$ as a mycobacterial 2-hydroxy-3-oxoadipate synthase. Chem. Biol. 17, 323–332
8. Bunik, V. L., and Fernie, A. R. (2009) Metabolic control exerted by the 2-oxoglutarate dehydrogenase reaction: a cross-kingdom comparison of the crossroad between energy production and nitrogen assimilation. Biochem. J. 422, 405–421
9. Wagner, T., Bellinzoni, M., Wehenkel, A., O’Hare, H. M., and Alzari, P. M. (2011) Functional plasticity and allosteric regulation of $\alpha$-ketoglutarate decarboxylase in central mycobacterial metabolism. Chem. Biol. 18, 1011–1020
10. Shim da, J., Nemeria, N. S., Balakrishnan, A., Patel, H., Song, J., Wang, J., Jordan, F., and Farinas, E. T. (2011) Assignment of function to histidines 260 and 298 by engineering the E1 component of the Escherichia coli 2-oxoglutarate dehydrogenase complex; substitutions that lead to acceptance of substrates lacking the 5-carboxyl group. Biochemistry 50, 7705–7709
11. Balakrishnan, A., Nemeria, N. S., Chakraborty, S., Kakalis, L., and Jordan, F. (2012) Determination of pre-steady-state rate constants on the Escherichia coli pyruvate dehydrogenase complex reveals that loop movement controls the rate-limiting step. J. Am. Chem. Soc. 134, 18644–18655
12. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254
13. Ellis, K. J., and Morrison, J. F. (1982) Buffers of constant ionic strength for studying pH-dependent processes. Methods Enzymol. 87, 405–426
14. de Souza, G. A., Mälen, H., Söfteland, T., Saelensminde, G., Prasad, S., Jonassen, I., and Wiker, H. G. (2008) High accuracy mass spectrometry analysis as a tool to verify and improve gene annotation using Mycobacterium tuberculosis as an example. BMC Genomics 9, 316
15. Kelkar, D. S., Kumar, D., Kumar, P., Balakrishnan, L., Muthusamy, B., Yadav, A. K., Srivastava, P., Marimuthu, A., Anand, S., Sundaram, H., Kingsbury, R., Harsha, H. C., Nair, B., Prasad, T. S., Chauhan, D. S., Katok, K., Katok, V. M., Chaerkady, R., Ramachandran, S., Dash, D., and Pandey, A. (2011) Proteogemonic analysis of Mycobacterium tuberculosis by high resolution mass spectrometry. Mol. Cell. Proteomics 10, M111 01627
16. Baykal, A., Chakraborty, S., Dodoo, A., and Jordan, F. (2006) Synthesis with good enantiomeric excess of both enantiomers of $\alpha$-ketols and acetalocats by two thiamin diphosphate-dependent decarboxylases. Bioorg. Chem. 34, 380–393
17. Vinogradov, M., Kaplin, A., Vyazmensky, M., Engel, S., Golbik, R., Tittmann, K., Uhlemann, K., Meshalkina, L., Barak, Z., and Chipman, D. M. (2005) Monitoring the acetoxydhydroxy acid synthase reaction and related carboligations by circular dichroism spectroscopy. Anal. Biochem. 342, 126–133
18. Cleland, W. W. (1979) Substrate inhibition. Methods Enzymol. 63, 500–513
19. O’Hare, H. M., Duran, R., Cervenansky, C., Bellinzoni, M., Wehenkel, A. M., Pritsch, O., Obal, G., Baumgartner, I., Vialaret, J., Johnson, K., and Alzari, P. M. (2008) Regulation of glutamate metabolism by protein kinases in mycobacteria. Mol. Microbiol. 70, 1408–1423
20. Nott, T. J., Kelly, G., Stach, L., Li, J., Westcott, S., Patel, D., Hunt, D. M., Howell, S., Buxton, R. S., O’Hare, H. M., and Smerdon, S. J. (2009) An intramolecular switch regulates phosphoenzyme-independent FHA domain interactions in Mycobacterium tuberculosis. Sci. Signal. 2, ra12
21. Balakrishnan, A., Gao, Y., Moorjani, P., Nemeria, N. S., Tittmann, K., and Jordan, F. (2012) Bipolarization of the thiamine diphosphate cofactor: assignment of tautomeric/ionization states of the 4’-aminopyrimidine ring when various intermediates occupy the active sites during the catalysis of pyruvate decarboxylase. J. Am. Chem. Soc. 134, 3873–3885
22. Kluger, R., and Pike, D. C. (1977) Active site generated analogues of reactive intermediates in enzymic reactions. Potent inhibition of pyruvate dehydrogenase by a phosphate analogue of pyruvatel. J. Am. Chem. Soc. 99, 4504–4506
23. Bunik, V. I., Denton, T. T., Xu, H., Thompson, C. M., Cooper, A. J., and Gibson, G. E. (2005) Phosphonate analogues of $\alpha$-ketoglutarate inhibit the activity of the $\alpha$-ketoglutarate dehydrogenase complex isolated from brain and in cultured cells. Biochemistry 44, 10552–10561
24. Nemeria, N., Chakraborty, S., Baykal, A., Korotchkina, L. G., Patel, M. S., and Jordan, F. (2007) The 4’-aminopyrimidine tautommer of thiamine diphosphate is poised for catalysis in asymmetric active centers on enzymes. Proc. Natl. Acad. Sci. U.S.A. 104, 78–82
25. Kaplin, A., Binshtein, E., Vyazmensky, M., Steinmetz, A., Barak, Z., Chipman, D. M., Tittmann, K., and Shaanan, B. (2008) Glyoxylate carboligase lacks the canonical active site glutamate of thiamine-dependent enzymes. Nat. Chem. Biol. 4, 113–118
26. de Carvalho, L. P., Fischer, S. M., Marrero, J., Nathan, C., Eht, S., and Rhee, K. Y. (2010) Metabolomics of Mycobacterium tuberculosis reveals compartmentalized co-carboligasi of carbon substrates. Chem. Biol. 17, 1122–1131
27. Tittmann, K., Vyazmensky, M., Hübler, G., Barak, Z., and Chipman, D. M.
Allosteric Regulation during Individual Catalytic Steps

(2005) The carbocation reaction of acetoxyhydroxycarbonyl synthase II: steady-state intermediate distributions in wild type and mutants by NMR. Proc. Natl. Acad. Sci. U.S.A. 102, 553–558
28. Porat, I., Vinogradov, M., Vyazmensky, M., Lu, C. D., Chipman, D. M., Abdelal, A. T., and Barak, Z. (2004) Cloning and characterization of acetoxyhydroxycarbonyl synthase from Bacillus steaothermophilus. J. Bacteriol. 186, 450–574
29. Chipman, D., Barak, Z., and Schloss, J. V. (1998) Biosynthesis of 2-aceto-2-hydroxy acids: acetolactate synthases and acetoxyhydroxycarbonyl synthases. Biochim. Biophys. Acta 1385, 401–419
30. Choi, K. J., Yu, Y. G., Hahn, H. G., Choi, J. D., and Yoon, M. Y. (2005) Allosteric Regulation during Individual Catalytic Steps.