Ability of Single-Site Mutants of Citrate Synthase To Catalyze Proton Transfer from the Methyl Group of Dethiaacetyl-Coenzyme A, a Non-Thioester Substrate Analog†

Linda C. Kurz,*,‡ James H. Roble,‡ Tanuj Nakra,‡ George R. Drysdale,‡ Jenny M. Buzan,‡ Benjamin Schwartz,§ and Dale G. Drueckhammer§

Department of Biochemistry and Molecular Biophysics, Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri 63110, and Department of Chemistry, Stanford University, Stanford, California 94305

Received December 12, 1996; Revised Manuscript Received February 5, 1997⊗

ABSTRACT: The catalytic strategies of enzymes (such as citrate synthase) whose reactions require the abstraction of the α-proton of a carbon acid remain elusive. Citrate synthase readily catalyzes solvent proton exchange of the methyl protons of dethiaacetyl-coenzyme A, a sulfur-less, ketone analog of acetyl-coenzyme A, in its ternary complex with oxaloacetate. Because no further reaction occurs with this analog, it provides a uniquely simple probe of the roles of active site interactions on carbon acid proton transfer catalysis. In view of the high reactivity of the analog for proton transfer to the active site base, its failure to further condense with oxaloacetate to form a sulfur-less analog of citryl-coenzyme A was unexpected, although we offer several possible explanations. We have measured the rate constants for exchange, k_{exch}, at saturating concentrations of the analog for six citrate synthase mutants with single changes in active site residues. Comparisons between the values of k_{exch} are straightforward in two limits. If the rate of exchange of the transferred proton with solvent protons is rapid, then k_{exch} equals the forward rate constant for proton transfer, and k_{exch} values for different mutants compare directly the rate constants for proton transfer. If the exchange of the transferred proton with protons in the bulk solution is the slow step and the equilibrium constant for proton transfer is unfavorable (as is likely), then k_{exch} equals the product of the equilibrium constant for proton transfer and the rate constant for exchange of the transferred proton with bulk solvent. If that exchange rate with bulk solution remains constant for a series of mutant enzymes, then k_{exch} values compare the equilibrium constants for proton transfer. The importance of the acetyl-CoA site residues, H274 and D375, is confirmed with D375 again implicated as the active site base. The results with the series of oxaloacetate site mutants, H320X, strongly suggest that activation of the first substrate, oxaloacetate, through carbonyl bond polarization, not just oxaloacetate binding in the active site, is required for the enzyme to efficiently catalyze proton transfer from the methyl group of the second substrate.

The catalytic strategies of enzymes whose reactions require the abstraction of the α-proton of a carbon acid have been the subject of much recent discussion and controversy (*vide infra*). The problem solved by these enzymes is both thermodynamic and kinetic in nature. Carbon acids are weak acids ($pK_a s \geq 20$), and the side chains of the basic amino acids are weak bases ($pK_a s \leq 8$). The rates of proton transfers from carbon acids are typically very slow in comparison to the rates from normal (heteroatom) acids of equal pK_a (intrinsic barriers for proton transfer from carbon acids are typically 9−12 kcal/mol greater than for normal acids, a factor of as much as 10^9 in rate; Gerlt & Gassman, 1993a,b). Short hydrogen bonds of unusual strength have been proposed to account for both the increased acidity and reactivity of these carbon acids in enzyme systems (Gerlt & Gassman, 1993a,b; Cleland & Kreevoy, 1995; Frey *et al*., 1994). The existence of such unusually short hydrogen bonds (Usher *et al*., 1994) and the demonstration of their unusual spectroscopic properties is undisputed (Frey *et al*., 1994). However, on both theoretical (Guthrie & Kluger, 1993; Warshel *et al.*, 1995; Warshel & Papazyan, 1996) and experimental grounds (Shan *et al*., 1996; Usher *et al*., 1994; Schwartz *et al*., 1995; Schwartz & Drueckhammer, 1995; Kato *et al.*, 1996), evidence has been presented which shows that the actual energies associated with formation of these short hydrogen bonds are not sufficient to account for the catalytic power of the enzymes and furthermore that more conventional electrostatic arguments will suffice. New experimental evidence supporting the importance of these hydrogen bonding interactions has recently been reported (Shan & Herschlag, 1996), and the issue is likely to remain unresolved for some time.

The reaction catalyzed by citrate synthase, the Claisen condensation of oxaloacetic acid (OAA)[1] with acetyl-CoA (AcCoA), requires proton transfer from the methyl group of the carbon acid, AcCoA, to an active site base (D375). High-resolution crystal structures are available of the ternary complexes of CS−OAA with dethiacarboxymethyl-CoA and carboxymethyl-CoA, analogs of the enol(ate) intermediate which is the product of the proton transfer (Usher *et al*.,

† Supported by National Institutes of Health Grant GM33851 (L.C.K.) and National Science Foundation Grant MCB9321371 (D.G.D.). Support for T.N. was provided in part by a grant to Washington University from the Howard Hughes Medical Institute through the Undergraduate Biological Sciences Education Program.

‡ Washington University School of Medicine.

§ Stanford University.

⊗ Abstract published in *Advance ACS Abstracts,* March 15, 1997.

S0006-2960(96)03058-9 CCC: $14.00

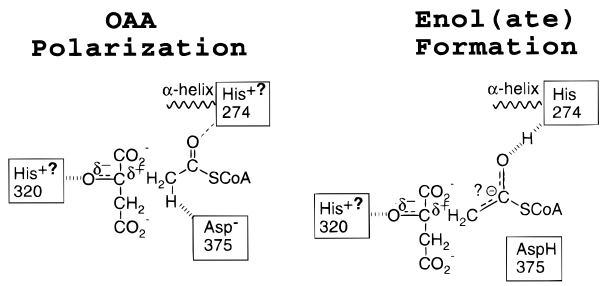


FIGURE 1: Working hypotheses for the roles of the residues studied in this work in the condensation reaction of citrate synthase.

1994). In both of these complexes, an unusually short H bond (OH···O distance < 2.4 Å) exists between the O atom mimicking the methyl carbon of AcCoA and the O atom of the carboxylate side chain of the active site base (D375). The proton in this hydrogen bond of these analog complexes has an unusually high proton chemical shift (A. McDermott *et al*., unpublished results from solid-state NMR experiments) similar in magnitude to that reported for the proton in the unusually short Asp-His H bond of the serine protease catalytic triad (Frey *et al*., 1994).

In an effort to further the understanding of the factors important to the enzymatic catalysis of proton transfers from carbon acids, we report here a study of the effects of several single-site mutations in the catalytic residues of citrate synthase (Alter *et al*., 1990) on the ability of the enzyme−OAA complexes to catalyze exchange of protons from the medium with the protons of the methyl group of dethia-AcCoA, a non-thioester substrate analog. The exchange of the methyl protons of dethia-AcCoA with solvent deuterons provides a uniquely simple probe; this analog undergoes no significant reaction in the active site other than this exchange of the protons of its methyl group. No detectable condensation occurs to produce a dethia analog of citryl-CoA or any citrate-like product (because of the substitution of a methylene group for the sulfur of CoA, any condensation product would be nonhydrolyzable).

An active site cartoon illustrating our working hypotheses for the roles of the residues studied in this work in the condensation reaction of citrate synthase is found in Figure 1. We find that changes in the AcCoA subsite residues D375 and H274 and the OAA subsite residue H320 severely impair the enzymes' abilities to catalyze proton transfer from the analog. The importance of D375 to the condensation half-reaction (in which proton transfer from AcCoA is required) has previously been questioned (Man *et al*., 1991), but its importance is again (Pereira *et al*., 1994) confirmed here. It is known that H320 forms a hydrogen bond to the carbonyl of OAA (Wiegand *et al*., 1984) and that it is necessary for the polarization/activation of the carbonyl (Kurz *et al*., 1995, unpublished). The deleterious effect of changes in H320 on the exchange rate dramatically illustrates that activation of the second substrate, AcCoA (in the preferred-ordered kinetic mechanism of CS), depends upon activation of the first substrate, OAA.

MATERIALS AND METHODS

Enzymes.[2] Crystalline citrate synthase from pig heart was obtained from Sigma Chemical Co. (St. Louis, MO).

Cloned Enzymes. A new construct for overexpression of the mature length protein has been developed, solving problems of low yields and proteolytic degradation experienced using previous procedures (Evans *et al*., 1988a,b; Kurz *et al*., 1992b)

Expression System. The vector we used was plasmid pJMB100A, a chimeric vector constructed in our laboratory and comprised of the minimal synthetic promoter region from pARC306A (M. Bittner, National Center for Human Genome Research, Rockville, MD), and the replicon, f1 origin, and ampicillin resistance marker from pMONIFABP (Sacchettini *et al*., 1990). pARC306A is a pUC-based plasmid with a synthetic *recA* promoter, a synthetic translational enhancer region based on the sequence of the T7 promoter 10 leader, a restriction site cluster, and transcription terminators at both ends of the expression cassette. pMONIFABP is a medium-copy number vector with an f1 origin for single-strand DNA synthesis, as well as a natural sequence *recA* promoter and T7 promoter 10 leader region. The Rec7 transcription unit from pARC306A was isolated as a *Pvu*II fragment and was ligated to the pMONIFABP *Acc*I-*Hin*dIII replicon fragment (blunt-ended and dephosphorylated). The resultant plasmid with the transcription cassette in the same orientation relative to the ampicillin resistance gene was named pJMB100A (Figure 2).

Strains. The following two strains were used: W620, *gal7* (*F*$^-$, λ^-, *glnV44*(AS), *gltA6*, *galK30*, *pyrD36*, *relA1*?, *rpsL129*, *thi-1*) from the *E. coli* Genetic Stock Center, and MG1655 (*F*$^-$, λ^-).

Recloning the CS Gene. The original expression clone of the CS gene had two extra codons at the amino terminus that were unrelated to CS and retained three of the signal sequence residue codons as well (Evans *et al*., 1988b). The expression system used a T7 promoter dependent on a heat inducible T7 RNA polymerase carried on a second plasmid. We removed the extra codons and created an NcoI site by selective amplification from the pT7-7PCS plasmid using the forward primer, 5′-GACCTCCATGGCTTCTTCCAC-GAAC-3′, which includes an initiating methionine codon within the *Nco*I site (indicated by underlining), followed by codons for the amino acid sequence ASSTN, which is the normal N terminus for mature PCS. The reverse primer for amplification was 5′-GGTAGAGATTCCGGTAG-3′ located downstream of a unique *Bgl*II site within the gene. As expected, the product of the amplification was a fragment 605 bp in length. This fragment was trimmed with *Nco*I and *Bgl*II and combined in a three-fragment ligation with the *Bgl*II-*Eco*RI fragment from unmodified pT7-7PCS, and the *Nco*I-*Eco*RI-digested pJMB100A, to obtain the full CS gene with the correct mature enzyme N terminus in the Rec7 promoter vector.

Mutants. Many of the mutations originally constructed in the two-plasmid CS expression system (Evans *et al.*,

[1] Abbreviations: AcCoA, acetyl-coenzyme A; CD, circular dichroism; CS, citrate synthase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); OAA, oxaloacetate; CMCoA, carboxymethyl-coenzyme A; CMX, carboxymethyldethia-CoA; dethia-AcCoA, dethiaacetyl-coenzyme A; WT, wild-type CS protein containing the naturally occurring amino acid sequence. The dethia analogs differ from carboxymethyl-CoA and acetyl-CoA because of replacement of the sulfur atom by a methylene group which forms a ketone. The dethia-AcCoA has also been called acetonylcarba(dethia)-CoA (Stewart & Wieland, 1978).

[2] Enzyme concentrations are given in terms of active sites (not the dimer).

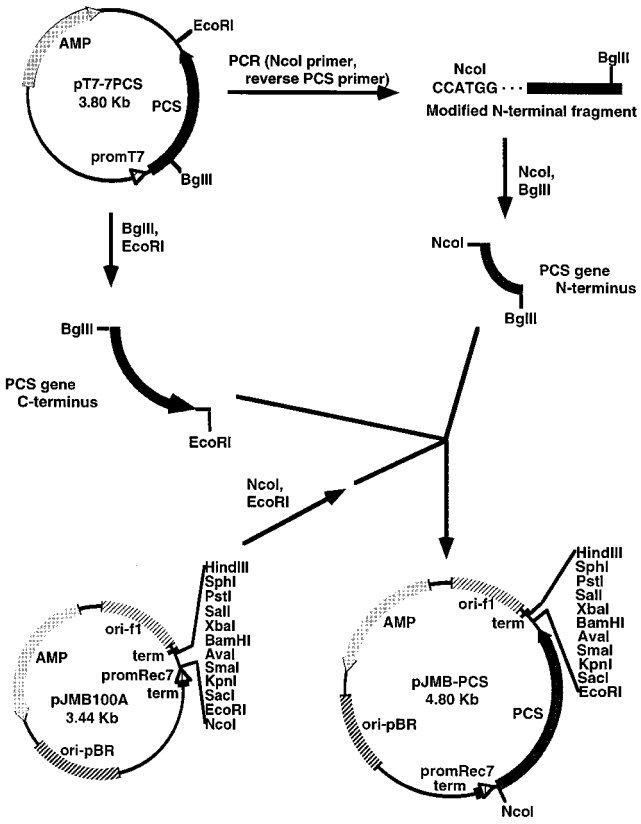


FIGURE 2: Flow chart of Construction of pJMB-PCS. The CS gene from pT7-7PCS was modified by selective amplification to correct the coding sequence for the N terminus of mature CS. pJMB-PCS was constructed by ligating the modified sequence with the remainder of the CS gene from pT7-7PCS and the vector pJMB100A. Refer to the text for details.

1988a,b) were transferred directly into the new system. Since each mutation of interest was contained between unique *Bgl*II and *Eco*RI sites of the CS gene locus, the *Bgl*II-*Eco*RI fragment from the pT7-7PCS plasmid with the mutation was simply exchanged with the *Bgl*II-*Eco*RI fragment of pJMB-PCS(WT). All mutations were confirmed by sequencing.

Mutant H320Q. The mutation H320Q was constructed in this lab using the Sculptor mutagenesis kit (Amersham), with the primer (complementary to the coding strand) 5′-CTTAGTACTGCCTGACCATAGCCTG-3′, where the underlined bases are mutations. The first mutation creates the glutamine codon, and the second is a silent mutation in the codon immediately upstream that destroys a *Bal*I restriction site, allowing rapid screening for mutant DNA. The mutagenesis reaction mixture was transformed directly into *gltA*$^-$ strain W620*gal7*, and ampicillin resistant colonies were screened for the inability to grow on minimal acetate plates, indicating that the CS gene on the acquired plasmid was inactive. The mutation was further identified by restriction analysis and DNA sequencing.

Substrates and Inhibitors. [2-^{13}C]OAA was prepared as described in Kurz *et al.* (1992a).

Dethiaacetyl-CoA (dethia-AcCoA) was prepared as described by Martin *et al.* (1994).

The dethia-AcCoA sample was desalted for experiments with the enzymes. Lyophilized fractions from the reverse phase HPLC column, 390 mg of potassium salts containing 17 μmol of dethia-AcCoA, were dissolved in the minimum amount of water (~2 mL). The sample was applied to a (1.5 cm × 29 cm, ~50 mL) column containing Sephadex G-10 and was eluted with water. Fractions (1.5 mL) were monitored for conductivity and absorbance at 260 nm. Salt-free fractions were pooled and lyophilized; fractions containing dethiaAcCoA but contaminated with salt were combined and lyophilized separately. The salt-containing dethia-AcCoA fractions were reprocessed in the same way. After a total of four processing cycles, 15 μmol of salt-free dethia-AcCoA was obtained. The pH of the combined fractions was adjusted to 7.5 prior to a final lyophilization.

Circular Dichroism. Spectra and titration data were collected and data analyzed as previously described (Kurz *et al*., 1992a).

^{13}C NMR. ^{13}C spectra were obtained at 150.7 MHz using a Varian Unity 600 spectrometer equipped with a 5 mm multinuclear probe. Proton-decoupled spectra were obtained using Waltz decoupling. The temperature of the sample was 10 °C. The sample buffer was 50 mM Tris-HCl and 1 mM EDTA at "pH" = 7.50 and included 25% D_2O (for internal lock) and 0.15 M acetonitrile (as internal chemical shift standard). The cyano resonance of the standard was assigned the value of 118.9 ppm. Other details were as described previously (Kurz *et al*., 1992a).

Proton NMR and Kinetics of Solvent Exchange of the Methyl Protons of Dethia-AcCoA Catalyzed by Citrate Synthase. The rate of exchange of the dethia-AcCoA methyl protons with solvent deuterons was measured by an NMR technique similar to that described by Srere (1967).

Enzyme samples were exchanged into the buffer for the experiment, 50 mM KPO_4 at pD 7.9 in 99.9% D_2O (low paramagnetic content, Cambridge Isotopes), by a minimum of five cycles of concentration (≤0.5 mL) and redilution (~3 mL) using centricon-30 (Amicon) centrifugal concentrators. The centricon concentrators were exhaustively prerinsed to remove the glycerol storage solution.

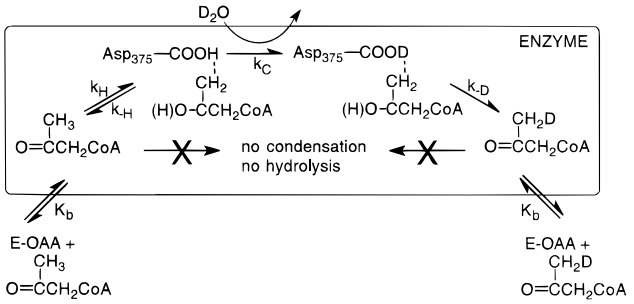
Proton spectra were obtained at 500 MHz using a Varian Unity-Plus 500 spectrometer equipped with a 5 mm reverse probe (Nalorac, Martinez, CA). Residual water signals were suppressed using transmitter presaturation (continuous wave decoupling centered on the water frequency) during the delay between acquisitions. The temperature of the sample was 25 °C. No chemical shift standard was included. Spectra were collected with a 90°, pulse and the delay between acquisitions was set to at least 5 times the T_1 value of the slowest relaxing proton of interest. The pantothenate methyl protons have short T_1 relaxation times of 0.41 and 0.47 s, and the ketone methyl protons have T_1s of 2.38 s. For each time point, 32 transients requiring 7 min were averaged. The time assigned to each spectrum was the middle of this acquisition sequence (i.e. 3.5 min after initiating the collection).

The fraction of dethia-AcCoA methyl protons remaining (F in eq 1) as a function of time (t in minutes in eq 1) is obtained from the ratio of the integrated areas of the acetyl−methyl resonance at 2.35 ppm relative to the pantothenate methyl resonance at 0.85 ppm (Figure 5, Scheme 1). In some samples with high protein concentrations, the adenine aromatic protons were used as the reference. Data were fit to eq 1 using nonlinear regression analysis.

$$F = F^0 e^{-k_{obs}t} \quad (1)$$

where adjustable parameters were F^0, the fraction at zero

Scheme 1



time,[3] and k_{obs}, the apparent exchange rate constant. The rate constant for exchange, k_{exch}, was calculated from eq 2

$$k_{exch} = \frac{3[\text{dethia-AcCoA}]k_{obs}}{[\text{E}]} \quad (2)$$

Kinetic Methods. Citrate synthase activity (Srere, 1969) with the normal substrates, OAA and AcCoA, was monitored through the reaction of the sulfhydryl product, CoA, with DTNB, producing TNB which absorbs at 412 nm ($\epsilon = 14.1$ mM^{-1}; Riddles *et al*., 1979). Absorbance data were collected using a Cary 3 spectrophotometer whose cell compartment was thermostated at 25 ± 0.1 °C. Data were collected at substrate concentrations close to those of the dethia-AcCoA concentration of the NMR samples (∼1 mM). These concentrations are close to saturating even for the mutant enzymes (H320X) with the highest K_ds for AcCoA and OAA (L. C. Kurz *et al.*, unpublished data).

RESULTS

New E. coli Expression System for Pig Citrate Synthase. We have used a new expression system for CS that avoids the heat induction step of the previously used system (Evans *et al*., 1988a,b) and produces CS with the correct amino terminus for the mature processed enzyme. The vector pJMB100A (Figure 2) has a synthetic *recA* promoter and terminators at each end of the expression cassette to reduce uninduced expression. Transcription is induced with nalidixic acid added to the culture medium.

For production of the CS protein, pJMB-PCS(WT) was initially grown in strain MG1655, which has an active *E. coli* CS (*gltA*$^+$). Following induction, activity more than 50 times the CS activity was found in the absence of the plasmid-encoded protein. The purified protein was assayed and found to have a specific activity within experimental error of the value obtained for CS isolated from pig heart. N-Terminal sequencing gave ASSTNLKDILADLIP, indicating that the initiating methionine residue is quantitatively removed and the remaining residues have the correct sequence for mature CS. Total amino acid analysis was also as expected for mature CS.

For further expression of the WT protein, and of all mutants, strain W620*gal7* was used. This strain carries a point mutation in *gltA*, the gene for *E. coli* CS, and has less than 5% of the activity found in *gltA*$^+$strains as determined in extracts from cells carrying no plasmid. Wildtype CS and all of the CS mutants that we have expressed in this system have given us average yields of 100−150 mg of purified protein from 4 L of bacterial culture, except D375G. In order to obtain soluble D375G protein from this system, it was necessary to coexpress the chaperonin GroE. We used the plasmid pGroESL which constitutively expresses the GroEL and GroES proteins and has a chloramphenicol resistance marker for maintenance of the plasmid (gift of A. Gatenby, DuPont). In this way, we were able to obtain 10 mg of purified protein and an additional 30 mg of partially purified protein from 4 L of the D375G mutant strain.

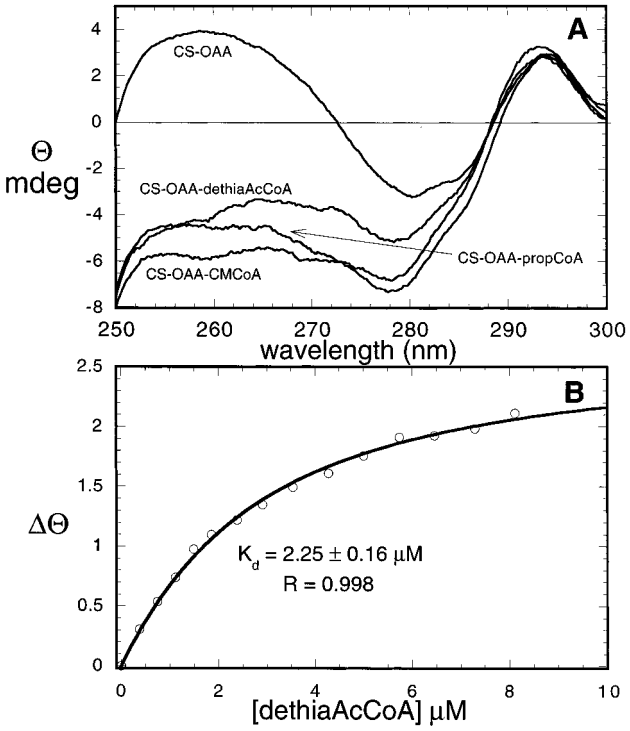
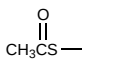
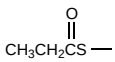
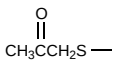
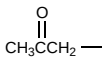
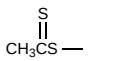
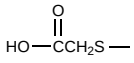


FIGURE 3: (A) High-wavelength UV CD spectra of CS-OAA, CS−OAA−propionyl-CoA, CS−OAA−dethia-AcCoA, and CS−OAA−CMCoA. (B) CD titration curve for WT CS with dethia-AcCoA at 256 nm. Titrations were done at 20 °C in 50 mM Tris-HCl and 0.2 mM EDTA at pH 7.50.

Circular Dichroism Spectra and Determination of the K_d for Dethia-AcCoA from the Ternary Complexes of CS−OAA−Dethia-AcCoA. Previously (Kurz *et al*., 1992a), we have observed large changes in the 230−300 nm CD spectrum upon formation of CS complexes with CoA analogs. The largest changes occur near 260 nm in the region of the CoA adenine absorbance. The relatively intense near-UV transition adenine absorption is subject to $\mu-\mu$ coupling with protein aromatic residues intensifying its very small intrinsic CD signal. For CS, the type of changes observed appear to be roughly characteristic of the binary or ternary nature of the CoA analog complex, with the largest changes occurring with ternary complexes. Figure 3A compares high-wavelength UV CD spectra for CS−OAA , CS−OAA−dethia-AcCoA, CS−OAA−propionyl-CoA, and CS−OAA−CMCoA. These changes may be used to measure the dissociation constants of the CoA analogs as described previously (Kurz *et al*., 1992a). Figure 3B shows the titration of the WT enzyme with dethia-AcCoA. The solid line is calculated from the results of nonlinear regression analysis of the data as in Kurz *et al*. (1992a). The K_d values

[3] To conserve the amount of dethia-AcCoA used for these measurements, successive additions of enzyme were made until a satisfactory exchange rate was obtained, resulting in some ambiguity as to the exact starting point of the measurement. The best zero time point was therefore determined by the regression analysis of the data.

Table 1: Properties of Ternary Complexes[a] of CoA Analogs with Citrate Synthase

analog	structure	K_d (μM)	k_{exch} (min^{-1})	k_{cat} (min^{-1})
AcCoA		OAA, 5[c]	none[e]	7000
		(*S*)-malate,[g] >1 mM	8×10^{-5}	na[i]
propionyl-CoA		5[b,c]	324 (*pro-S* proton)[b]	0.25[b]
acetonyl-CoA		1.0[d,h]	1.5[d,h]	no product detected
dethia-AcCoA		2.3	1573	no product detected
dithia-AcCoA[6]		53[c]	8 and 32	0.024
		(pH 7.5)	(pH 7.9 and 7.2)	(pH 7.5)
CMCoA		0.023[d]	na	na

[a] The ternary complex is the analog with CS−OAA except where it is CS−(*S*)-malate. [b] Weidman and Drysdale (1979). [c] K_m. [d] Kurz *et al.* (1992a). [e] Eggerer (1965). [f] Wlassics and Anderson (1989). [g] Exchange rate constant calculated from the data of Srere (1967) at 11 mM (*S*)-malate and 190 mM AcCoA. It proved impossible to saturate the WT enzyme with either malate or AcCoA (Eggerer, 1965), so comparisons of WT and mutants would have an unknown contribution from the likely differences of affinity of AcCoA and malate for the various proteins. [h] The protons of the methylene between the carbonyl and the sulfur exchange rapidly and nonenzymatically. [i] na = not applicable.

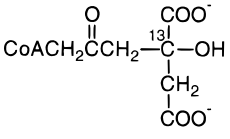
Table 2: Dissociation Constants, Exchange Rate Constants for Dethia-AcCoA, and Turnover Numbers with the Natural Substrate AcCoA for OAA Complexes of WT and Active Site Mutants

enzyme	K_d[a] (μM)	k_{exch} (min^{-1})	k_{cat}^{AcCoA} (min^{-1})
WT	2.3	1573	3300
AcCoA site mutants			
D375E	0.49	27	10
D375G	≤0.01	≤0.02	0[c]
H274G	1.9	0.35	1.0
OAA site mutants			
H320G	9.0	0.76	12
H320Q	14.0	1.12	33
H320N	10.43	0.16	1.4

[a] Determined by CD titration of the enzyme−OAA complex with dethia-AcCoA. [b] In 50 mM potassium phosphate/D_2O buffer at pD 7.9 used for the exchange reactions, the k_{cat} value for all the enzymes was found to be reduced about 2-fold from the value found under standard conditions. [c] See text.

for dissociation of dethia-AcCoA from the OAA complexes of the normal and mutant CS are reported in Table 2.

Search for a Condensation Product with the Carbon Spectrum of Ternary Complex of CS−[2-^{13}C]OAA−Dethia-AcCoA. The acidity of the methyl protons of the AcCoA thioester should be about the same (or even slightly less) than that of the methyl ketone analog (p$K_a \sim 20$; Amyes & Richard, 1992). It would seem reasonable then to expect the enzyme to catalyze condensation of the methyl group of the dethia analog with OAA as well as it does with AcCoA, although the resulting dethiacitryl-CoA would not be hydrolyzable. In accord with the known tight binding of citryl-



dethiaCitryl-CoA

CoA (Pettersson *et al.*, 1989; Kurz *et al.*, 1995), the dethiacitryl-CoA product might not be released from the enzyme into solution.

In order to investigate whether a bound condensation product is actually made, we obtained ^{13}C NMR spectra of solutions of [2-^{13}C]OAA−CS (carbonyl label) and dethia-AcCoA. If dethiacitryl-CoA were produced, we would expect a new resonance to appear close to that of the ^{13}C-OH of citrate. Figure 4A is the natural abundance carbon spectrum of citrate showing that the alcohol carbon resonates at 75.21 ppm. Figure 4B shows the spectrum obtained from a mixture of CS binary and ternary complexes. (The solution contains ≤40% of the binary CS−OAA and ≥60% of the ternary CS−OAA−dethia-AcCoA complexes.[4]) The OAA carbonyl in the ternary complex resonates at 206.2 ppm, close to the value obtained in the complex with the intermediate analog CMCoA and higher than that observed with another ketone substrate analog, acetonyl-CoA. The signal from the binary complex in this spectrum which is present at a concentration of about 0.3 mM demonstrates the sensitivity of the method. The binary CS−OAA and ternary complex spectra from a higher-concentration experiment, 2.5 mM, are shown in parts D and C of the figure, respectively. The solution for this experiment contained previously observed contaminants. The G-10 column routinely (Kurz *et al.*, 1992a,b) used to purify the binary complex was slightly too small for the higher concentrations of this experiment, and contaminants of the OAA preparation are evident as sharp (unbound) lines near 201 and 75 ppm (labeled "X" in the spectra). The size of the contaminant peaks already present in the binary complex remains unchanged in the ternary complex. The absence of a new resonance in the vicinity of 75 ppm in the ternary complex with dethia-AcCoA indicates that no appreciable amount of dethiacitryl-CoA is formed. We estimate that less than 5% of the [2-^{13}C]OAA carbonyl carbon is converted to an alcoholic carbon in the ternary complex with dethia-AcCoA.

[4] OAA is slowly lost from the CS−OAA binary complex. The appearance of pyruvate from the decarboxylation of OAA released from the binary complex becomes increasingly evident as time passes. (On the other hand, the OAA in ternary complexes is stable for months, *vide infra.*) As a consequence, some of the concentrations of species in NMR samples are only semiquantitative.

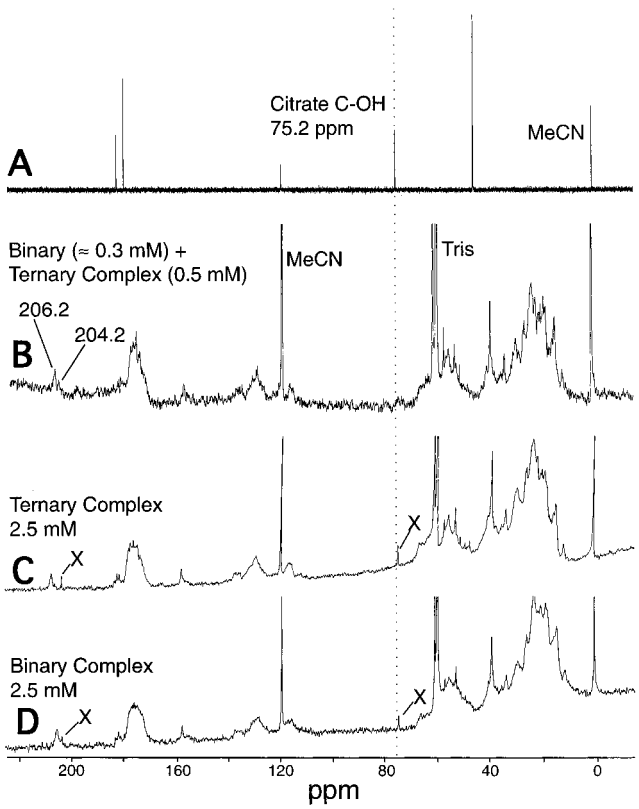


FIGURE 4: ^{13}C-NMR spectra of citrate and the binary and ternary complex of citrate synthase with [2-^{13}C]OAA and dethia-AcCoA: (A) 0.5 M citrate (spectrum at natural abundance), 1000 transients; (B) CS−[2-^{13}C]OAA (≈0.3 mM), CS−[2-^{13}C]OAA−dethia-AcCoA (0.5 mM), 30 000 transients (15 h); (C) CS−[2-^{13}C]OAA−dethia-AcCoA (2.5 mM), 36 000 transients (18 h); and (D) CS−[2-^{13}C]OAA (2.5 mM), 13 000 transients (6 h). Other conditions are described in Materials and Methods. The sharp resonances marked with an X are contaminants of the OAA preparation.

No changes with time could be detected in these ternary complex spectra even after storing the samples for 4 weeks at 4 °C, demonstrating the absence of slow formation of products.

Solvent Exchange of the Methyl Protons of Dethia-AcCoA Catalyzed by Citrate Synthase and Single-Site Mutants. Figure 5 shows the methyl region of the proton spectrum of dethia-AcCoA as a function of time after the addition of OAA and WT to the solution. The two methyl resonances on the right of the spectrum are the pantothenate methyls, while the sharp peak on the left is the ketone methyl. As exchange occurs, notice the appearance of isotope-shifted protons in the ketone methyl corresponding to dethia-AcCoA containing first one (10 and 31 min) and then two deuterons (shoulder visible at 58 min). Finally, the proton signal disappears when exchange is complete as shown in the top spectrum. Figure 6 shows the progress curve obtained for exchange catalyzed by H320G. The solid line is the nonlinear regression fit to eq 1. It is somewhat surprising that there is so little evidence of deviation from first-order (as a consequence of the reduction of the statistical factor and of the presence of a secondary isotope effect) as the dethia-AcCoA becomes more highly deuterated.

Exchange rate constants for WT and several single-site mutants are given in Table 2 and compared to the turnover numbers, k_{cat}^{AcCoA}, for reaction with AcCoA under the same experimental conditions.

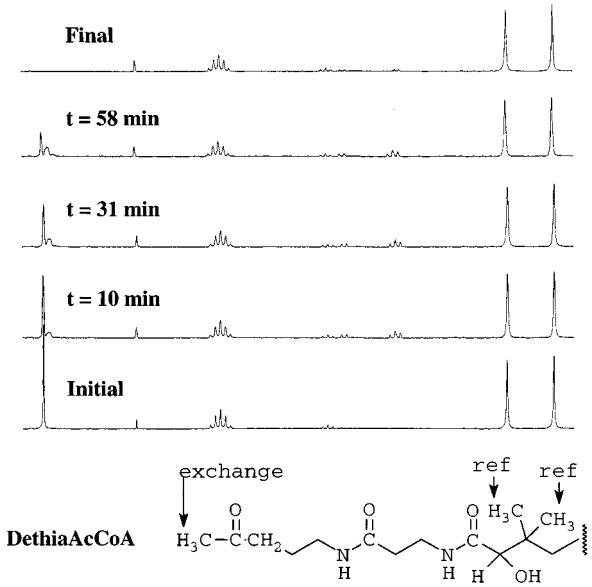


FIGURE 5: Methyl region of the proton spectrum of dethia-AcCoA as a function of time in the presence of citrate synthase. With 0.87 mM dethia-AcCoA, 0.6 mM OAA, and 0.015 μM CS, in 50 mM KPO_4 at pD 7.9. The initial spectrum was taken before adding OAA and CS. The final spectrum was obtained in a separate experiment with 4 μM CS. Low-molecular weight contaminants between the reference and ketone methyl peaks are apparent in spectra of solutions containing OAA and enzyme. The size of contaminant peaks does not change with time. (bottom) Partial structure of dethia-AcCoA showing the reference and exchanging methyl protons.

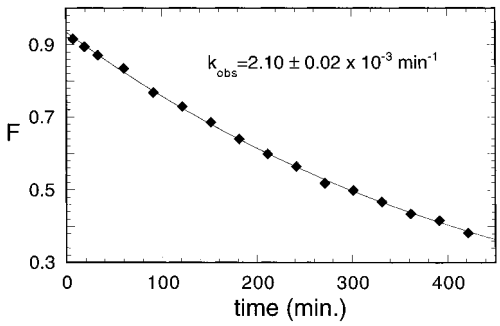


FIGURE 6: Determination of k_{obs} for exchange of the methyl protons with solvent deuterons catalyzed by H320G. With 0.96 mM dethia-AcCoA, 1.0 mM OAA, and 8.8 μM H320G.

DISCUSSION

New E. coli Expression System for Pig Citrate Synthase. Although previous attempts to express the mature CS protein with its correct N terminus were unsuccessful, it was readily synthesized in this expression system. Each mutant gene has been reanalyzed for its optimal induction conditions and its level of solubility. We have now been able to obtain large amounts of recombinant protein from six different mutant CS genes.

Internal Equilibrium Constant of the Condensation Step. In solution, the overall favorable equilibrium for the citrate synthase reaction ($K_{eq} \sim 1 \times 10^6$ at pH 7 and 38 °C; Stern *et al.*, 1952; Guynn *et al.*, 1973) is thermodynamically driven by the favorable free energy change for the hydrolysis of the thioester [$K_{eq} \sim 1 \times 10^6$ M at pH 7 (Guynn *et al.*, 1973) for acetyl-CoA hydrolysis]. In solution then, the value of the equilibrium constant for the condensation reaction must be about 1 M^{-1}.

On the enzyme, the reaction takes place with the direct product of the condensation reaction, citryl-CoA, remaining bound to the enzyme as an intermediate. Citryl-CoA is hydrolyzed in a step subsequent to the condensation. (Eggerer & Remberger, 1963; Srere, 1963). Extrapolation of the free solution thermodynamic data to a convincing estimate of the values for the enzyme internal equilibrium constants for either condensation or hydrolysis steps is not straightforward. For the condensation, the difference in molecularity between the enzyme bound versus the free solution reaction could conceivably lead to a large factor favoring condensation in the enzyme-bound reaction. The costly entropy requirement to bring AcCoA and OAA together in the correct orientation to react has presumably already been paid in binding energy (Jencks, 1975). However, changes from free solution values for the activities of water and other species which participate in the reactions in the active site are difficult to estimate. Using the partitioning of citryl-CoA between reversal of condensation and hydrolysis, as well as measured kinetic constants for the citrate synthase reaction, Pettersson *et al*. (1989) estimated an internal equilibrium constant of 0.74 for the condensation step. Indeed, the requirement of balanced internal equilibria for optimized catalysis (in which the internal equilibrium constant approaches a value of 1.0) would seem to be easily obtainable for the condensation step of citrate synthase (Burbaum *et al*., 1989).

Thus, our failure to find any detectable condensation product for the dethia (ketone) analog seems unexpected on theoretical and experimental grounds. However, the reactions catalyzed by citrate synthase are sensitive to steric factors (some examples are the poor activity of propionyl-CoA and the failure of α-keto acids other than OAA to serve as substrates). The difference in bond lengths and angles between the C−S−C of the natural thioester substrate and the C−C−C of the ketone analog may cause the "effective concentrations" in the analog complex to be too low to observe reaction. In addition, chemical factors favor the reactivity of the thioester enol(ate) over the corresponding species from the analog. The greater acidity of the ketone methyl protons (Amyes & Richard, 1992) is likely to be accompanied by a decrease in the nucleophilicity of its conjugate base in comparison with that from the thioester, and the presence of the polarizable sulfur atom in the thioester enol(ate) will increase its reactivity over that from the ketone even further (Jencks & Carriuolo, 1960). One or more of these factors must outweigh the greater concentration of enol(ate) present in the ketone analog complex. The foregoing arguments concerning the forward rate for condensation might reasonably be applied to the corresponding equilibrium constant as well.

Figure 3A compares the high-wavelength CD spectra of three ternary complexes of CS−OAA with CoA analogs: dethia-AcCoA, propionyl-CoA, and CMCoA. The primary origin of these signals lies in the CD induced in the nonchiral *adenine* chromophore (*vide supra*). The protein side chains of tyrosine and phenylalanine also contribute to the signal in this region. The CS−OAA−CMCoA complex is an analog of the reactive enol−enolate intermediate complex (Kurz *et al*., 1992a,b). The CS−OAA−propionyl-CoA complex is a stable ground-state complex, although it is competent for condensation because methyl citrate is slowly produced (Weidman & Drysdale, 1979). As shown by the differences in their CD spectra, it is clear that differences must exist in the structures of these complexes. Whether these structural differences can be translated into an understanding of their differing properties and reactivities will have to await a determination of their X-ray structures. Unfortunately, attempts to obtain crystals of the dethia-AcCoA−OAA−CS complex have so far been unsuccessful (S. J. Remington, personal communication).

Suitability of Dethia-AcCoA as a Probe of Factors Affecting Catalysis of Proton Transfer from AcCoA. Under the conditions used for its measurement (D_2O, phosphate buffer, and high dethia-AcCoA concentrations), the value of k_{exch} for the dethia analog is within a factor of 2 of the value of k_{cat} measured under the same conditions (Table 2). While the enzyme catalyzes proton exchange with a number of other CoA analogs (Table 1), none is as active as dethia-AcCoA. The next best analog is the poor substrate, propionyl-CoA, whose *pro-S* proton exchanges about $^1/_3$ as fast. Note the extremely slow exchange rate constant for another ketone CoA analog, acetonyl-CoA, which differs from dethia-AcCoA by the addition of a S atom. The exchange rate for AcCoA itself obtained with the OAA analog, (*S*)-malate, is slower still. Taken together, these data illustrate the sensitivity of the exchange rate constant to the details of the interactions within the active site.

For dethia-AcCoA, the lack of any significant reaction but exchange eliminates many of the complications of interpretation. For example, AcCoA exchange catalyzed by the CS−OAA complexes depends upon the actual proton transfer and its reverse, the release of the exchanged substrate, and the rate of further reaction of the intermediate to give products. This complication is well illustrated by the results for the WT enzyme where no exchange can be detected in the methyl group of AcCoA but easily measured exchange is evident in the methylene of citrate derived from the methyl of AcCoA (Marcus & Vennesland, 1958; Myers & Boyer, 1984) (AcCoA and citrate exchange data for the mutant enzymes will be reported in a subsequent publication). Presumably, the exchanged AcCoA reacts before significant amounts can be released back into the solution. While we cannot rule out a small amount of condensation of OAA with dethia-AcCoA, its presence will not complicate our conclusions in any major way.

Interpretation of the Specific Rate Constant for Exchange in Terms of Rate Constants of Elementary Steps. The exchange of the methyl protons of the dethia-AcCoA analog is the consequence of three processes as indicated in Scheme 1: (1) binding of the analog with the enzyme, (2) proton transfer to and from the active site base, and (3) exchange of the proton of the active site base with the bulk solvent (D_2O). For a three-site exchange, it is not possible to write a simple expression for a first-order rate constant in terms of the rate constants for these three processes because the statistical factor changes with time. In addition, derivation of simple expressions also requires that the dissociation rate constant for substrate and product (exchanged substrate) be rapid, i.e. that binding is adequately described as a prior equilibrium. If this is not the case, multiple exchanges could occur as a consequence of a single binding event. For low extents of exchange, limited to a single site, simple expressions are possible (*vide infra*).

Assuming normal association rate constants for dethia-AcCoA, the description of binding as a prior equilibrium

seems valid (Table 2). At saturating concentrations of substrate analog, the equilibrium constant for the binding step (K_b in Scheme 1) will not appear in the expression for the rate constant for exchange. The values of the dissociation constants (Table 2) of the analog from all the enzyme−OAA complexes in this study indicate that concentrations are close to saturating for all the NMR exchange experiments.

First consider the common assumption [as in Wlassics and Anderson (1989)] that the exchange of the proton extracted by the active site base with protons in the bulk solvent (k_C in Scheme 1) is very fast. In this case,

$$k_{exch}^{obs} = k_H \tag{3}$$

by inspection. Thus, if bulk solvent exchange of the transferred proton is fast, a comparison of the specific exchange rate constants for different mutants is a direct comparison of these enzymes' abilities to catalyze proton transfer from the methyl carbon of the analog.

To obtain the other limit, we can make the steady-state assumption for the enzyme-bound intermediates in Scheme 1, obtaining for the initial velocity of exchange

$$\frac{d[P]/dt}{[E_T]} = k_{exch}^{obs} = \frac{k_H k_{-D} k_C}{k_{-D}(k_C + k_{-H}) + k_H(k_C + k_{-D})} \tag{4}$$

In this limit, $k_C \ll k_{-D}$ and k_{-H} (which, incidentally, is required for the validity of the steady-state approximation, since the concentration of the intermediate linked by the irreversible steps described by k_C and k_{-D} must be low) so that then

$$k_{exch}^{obs} = \frac{k_H k_C}{k_{-H} + k_H} \tag{5}$$

If the equilibrium constant for formation of the activated intermediate is sufficiently unfavorable (which is likely) so that $k_{-H} \gg k_H$, then the expression simplifies further to

$$k_{exch}^{obs} = \frac{k_H}{k_{-H}} k_C \tag{6}$$

In this case, comparison of the values of exchange rate constants for different mutants is a comparison between the product of the rate constant for the exchange of the removed proton with bulk solvent (presumably limited by the rate of the open/closed conformation change) and the equilibrium constant for formation of the intermediate.

Thus, if we can successfully argue in any case either that k_C is not kinetically significant or that it is not greatly changed by a given mutation, then a comparison of k_{exch} for different mutants compares either the rate constant or the equilibrium constant for formation of the conjugate base of the dethia-AcCoA carbon acid.

AcCoA Binding Site Mutants D375E and H274G. Of the two conserved AcCoA site residues, H274 and D375, early workers suggested (on the basis of consideration of free solution reactivities and early X-ray structures) that the imidazole side chain of H274 was likely to be the base participating in proton transfer from the methyl of AcCoA and that D375 plays some other role (Wiegand & Remington, 1986). However, later crystallographic studies (Karpusas *et al*., 1990, 1991) indicated that the carboxylate side chain of D375 is almost certainly the base that removes the proton from AcCoA in the condensation reaction (Figure 1). However, Man *et al*. (1991), in their study of the Glu mutant of the residue corresponding to D375 in the *E. coli* enzyme, suggested that the primary role of D375 is in the hydrolysis reaction and that H274 is the base removing the methyl proton. While we have also confirmed the importance of D375 for the hydrolysis reaction of the pig heart enzyme (L. C. Kurz *et al*., unpublished results), the low value of k_{exch} (Table 2) for the D375E mutant also affirms the importance of this residue to the condensation reaction.

H274 is also important as indicated by the low value of k_{exch} obtained for H274G. The X-ray structures of both CS−(*S*)-malate−AcCoA complexes (Karpusas *et al*., 1991) and CS−OAA complexes with the enol−enolate analogs, CMX and CMCoA (Usher *et al*., 1994; Karpusas *et al*., 1990), show a hydrogen bond between the carbonyl oxygen of the AcCoA analog and the H274 ND1. The H274 imidazole side chain lies at the amino terminus of a long α-helix (positive end of the macrodipole) and furthermore the hydrogen bonding pattern strongly suggests that the side chain is neutral in these complexes. This unusual interaction has led to the suggestion that there may be actual proton transfer to generate an AcCoA enol−imidazolate intermediate. Whatever the resolution of these controversial proposals, this interaction is clearly functionally important.

While we have categorized H274 as primarily an AcCoA site residue, it also interacts with the OAA site as is apparent in structure (Wiegand & Remington, 1986) and solution studies (Kurz *et al*., 1992b). NMR evidence for two forms of the binary OAA−H274G complex is found in solution. The ternary complex with CMCoA in this mutant fails to show both the change in the ^{13}C chemical shift of the carboxylate carbon of CMCoA which accompanies the formation of the short hydrogen bond present in the wild-type enzyme ternary complex and the chemical shift change of the OAA carbonyl carbon which signifies its polarization.

If both AcCoA site residues are important, which is the catalytic base? We reason that removal of the catalytic base should have a more deleterious effect on the enzyme than removal of a residue primarily involved in hydrogen bonding interactions with substrates and intermediates. H274G has measurable activity, while we have been unable to convincingly demonstrate citrate synthase activity with D375G. D375G actually shows a greater rate in the standard DTNB assay in the absence of OAA than it does in its presence. (This result could indicate either a substrate-induced protection of the protein's reactive sulfhydryl or a misdirected hydrolysis of AcCoA.) We are unable to distinguish these possibilities from the naturally occurring reaction. The protein concentrations required to observe a rate are too high to use the disappearance of the thioester absorbance (233 nm) and the rate too slow to demonstrate product formation by either enzymatic or radiochemical methods (L. C. Kurz *et al*., unpublished). The high affinity of D375G for the substrates, as well as for the intermediate-state analog, CMCoA, has been reported previously (Kurz *et al*., 1992b). It seems most likely then that D375 is the base in the citrate synthase condensation reaction as indicated by the crystal studies. As would be expected for removal of the catalytic base, the exchange reaction for D375G is barely detectable (Table 2) and the slight rate observed may be a consequence of nonspecific catalysis.

OAA Binding Site Mutants H320X. H320 forms a hydrogen bond with the carbonyl of OAA (Wiegand *et al*., 1984). H320 is also thought to be the source of the proton of the alcohol of citrate (Wiegand & Remington, 1986). Polarization of the carbonyl of OAA, increasing the positive charge density at the carbon, is an essential part of the catalytic strategy of citrate synthase. By increasing the electrophilicity of the carbonyl carbon, the enzyme increases its reactivity toward condensation with the nucleophilic center generated in the methyl carbon of AcCoA (Kurz *et al*., 1985; Kurz & Drysdale, 1987). Substitution of glycine for histidine at this position results in an enzyme unable to polarize the carbonyl of OAA (Kurz *et al*., 1995). Replacement of this histidine residue with asparagine, glutamine, or arginine also results in enzymes equally incapable of OAA carbonyl polarization (L. C. Kurz *et al*., unpublished).

For this series of mutants, H320X (X = G, N, or Q), computer modeling studies in which the new side chain has been substituted for the naturally occurring one in both open and closed structures do not reveal any differential effects on either the number of unfavorable contacts or favorable interactions lost between the two conformational forms of the enzyme (J. S. Remington, personal communication). While this does not eliminate the possibility that the value of k_C differs for different mutants, it does suggest that these effects will not be major.

So even in the case where the access of the active site base to bulk solvent is slow, a comparison between mutants and wild type reflects the relative stability of the reactive intermediate. If k_C is not rate-limiting, then k_{exch} compares the proton transfer rate constants directly.

Several lines of evidence have indicated the importance of the binding of OAA to the overall catalysis of the citrate synthase reaction. The kinetic reaction is of the preferred-ordered type with OAA binding first (Johansson & Pettersson, 1974). The binding of OAA initiates a conformation change to a partially closed form which becomes completely closed, excluding bulk solvent from the active site, upon the binding of AcCoA (Wiegand & Remington, 1986). NMR studies of H320X−OAA [H320G in Kurz *et al*. (1995) and H320N, H320Q, and H320R in Kurz *et al*. (unpublished)] complexes with the analog of the enol(ate) intermediate, CMCoA, fail to show the changes in the chemical shift of the CMCoA carboxylate carbon which are observed in the wild-type enzyme complexes. At a minimum, these NMR results show a perturbation in the hydrogen bonding interactions at the AcCoA binding site which are induced by events at the OAA site.

Noting (Table 2) the low values of k_{exch} for all the mutants, H320X, we conclude that activation of OAA, not just its binding in the active site, is required for the enzymes' ability to catalyze efficient proton transfer from AcCoA. This conclusion is further supported by the extremely low rate of exchange induced by the OAA analog, (*S*)-malate, which contains an alcohol rather than a carbonyl carbon at the 2 position.

The failure of H320X mutants to activate OAA and AcCoA does not arise from a gross failure of the mutants to undergo the "open" to "closed" conformation change (except possibly for H320R where the steric bulk of R is considerably greater than the substitutions in the other members of the series). Ternary complexes of H320X (except H320R) have near-UV CD spectra with the characteristically large induced CD in the adenine chromophore similar to but not identical to those of WT ternary complexes with CoA analogs (data not shown). The ^{13}C resonance of the carboxyl of the enol(ate) analog inhibitor moves from the fast to slow exchange regime (irrespective of any change in the value of its chemical shift) upon formation of the ternary OAA−CMCoA complex from the binary CMCoA complex (Kurz *et al*., 1995, unpublished). Recent low-angle neutron scattering experiments (S. Henderson *et al*., unpublished) show that H320G undergoes a reduction in its radius of gyration consequent to formation of its OAA−CMCoA ternary complex similar in magnitude to that shown by the WT enzyme. Thus, the structural basis of the failure of H320X to efficiently catalyze proton transfer from the AcCoA carbon acid will have to be sought in X-ray studies of these mutants and their various complexes.

REFERENCES

Alter, G. M., Casazza, J. P., Zhi, W., Nemeth, P., Srere, P. A., & Evans, C. (1990) *Biochemistry 29*, 7557.

Amyes, T. L., & Richard, J. P. (1992) *J. Am. Chem. Soc. 114*, 10297.

Burbaum, J. J., Raines, R. T., Albery, W. J., & Knowles, J. R. (1989) *Biochemistry 28*, 9293.

Cleland, W. W., & Kreevoy, M. M. (1994) *Science 264*, 1887.

Eggerer, H. (1965) *Biochem. Z. 343*, 111.

Eggerer, H., & Remberger, U. (1963) *Biochem. Z. 337*, 202.

Evans, C. T., Owen, D. D., Slaughter, C. A., & Srere, P. A. (1988a) *Biochem. Biophys. Res. Commun. 157*, 1231.

Evans, C. T., Owens, D. D., Sumegi, B., Kispal, G., & Srere, P. A. (1988b) *Biochemistry 27*, 4680.

Frey, P. A., Whitt, S. A., & Tobin, J. B. (1994) *Science 264*, 1927.

Gerlt, J. A., & Gassman, P. G. (1993a) *J. Am. Chem. Soc. 115*, 11552.

Gerlt, J. A., & Gassman, P. G. (1993b) *Biochemistry 32*, 11943.

Guthrie, J. P., & Kluger, R. (1993) *J. Am. Chem. Soc. 115*, 11569.

Guynn, R. W., Gelberg, H. J., & Veech, R. L. (1973) *J. Biol. Chem. 248*, 6957.

Jencks, W. P. (1975) *Adv. Enzymol. 43*, 219.

Jencks, W. P., & Carriuolo, J. (1960) *J. Am. Chem. Soc. 82*, 1778.

Johansson, C-J., & Pettersson, G. (1974) *Eur. J. Biochem. 42*, 383.

Karpusas, M., Branchaud, B., & Remington, S. J. (1990) *Biochemistry 29*, 2213.

Karpusas, M., Holland, D., & Remington, S. J. (1991) *Biochemistry 30*, 6024.

Kato, Y., Toledo, L. M., & Rebek, J., Jr. (1996) *J. Am. Chem. Soc. 118*, 8575.

Kurz, L. C., & Drysdale, G. R. (1987) *Biochemistry 26*, 2623.

Kurz, L. C., Ackerman, J. J. H., & Drysdale, G. R. (1985) *Biochemistry 24*, 452.

Kurz, L. C., Shah, S., Crane, B. R., Donald, L. J., Duckworth, H. W., & Drysdale, G. R. (1992a) *Biochemistry 31*, 7899.

Kurz, L. C., Drysdale, G. R., Riley, M. C., Evans, C. T., & Srere, P. A. (1992b) *Biochemistry 31*, 7908.

Kurz, L. C., Shah, S., Frieden, C., Nakra, T., Stein, R. E., Drysdale, G. R., Evans, C. T., & Srere, P. A. (1995) *Biochemistry 34*, 13278.

Man, W.-J., Yan, L., O'Connor, C. D., & Wilton, D. C. (1991) *Biochem. J. 280*, 521.

Marcus, A., & Vennesland, B. (1958) *J. Biol. Chem. 233*, 722.

Martin, D. P., Bibart, R. T., & Drueckhammer, D. G. (1994) *J. Am. Chem. Soc. 116*, 4660.

Myers, J. A., & Boyer, P. D. (1984) *Biochemistry 23*, 1264.

Pereira, D. S., Donald, L. J., Hosfield, D. J., & Duckworth, H. W. (1994) *J. Biol. Chem. 269*, 412.

Pettersson, G., Lill, U., & Eggerer, H. (1989) *Eur. J. Biochem. 182*, 119.

Riddles, P. W., Blakeley, R. L., & Zerner, B. (1979) *Anal. Biochem. 94*, 75.

Sacchettini, J. C., Banaszak, L. J., & Gordon, J. I. (1990) *Mol. Cell. Biochem. 98*, 81.
Schwartz, B., & Drueckhammer, D. G. (1995) *J. Am. Chem. Soc. 117*, 11902.
Schwartz, B., Drueckhammer, D. G., Usher, K. C., & Remington, S. J. (1995) *Biochemistry 34*, 15459.
Shan, S., & Herschlag, D. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 14474.
Shan, S., Loh, S., & Herschlag, D. (1996) *Science 272*, 97.
Srere, P. A. (1963) *Biochim. Biophys. Acta 77*, 693.
Srere, P. A. (1967) *Biochem. Biophys. Res. Commun. 26*, 609.
Srere, P. A. (1969) *Methods Enzymol. 14*, 3.
Stern, J. R., Ochoa, S., & Lynen, F. (1952) *J. Biol. Chem. 198*, 313.
Stewart, C. J., & Wieland, T. (1978) *Liebigs Ann. Chem.*, 57.
Usher, K. C., Remington, S. J., Martin, D. P., & Drueckhammer, D. G. (1994) *Biochemistry 33*, 7753.
Warshel, A., & Papazyan, A. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 13665.
Warshel, A., Papazyan, A., & Kollman, P. A. (1995) *Science 269*, 102.
Weidman, S. W., & Drysdale, G. R. (1979) *Biochem. J. 177*, 169.
Wiegand, G., & Remington, S. (1986) *Annu. Rev. Biophys. Biophys. Chem. 15*, 97.
Wiegand, G., Remington, S., Deisenhofer, J., & Huber, R. (1984) *J. Mol. Biol. 174*, 205.
Wlassics, I. D., & Anderson, V. E. (1989) *Biochemistry 28*, 1627.

BI963058S