Effects of Changes in Three Catalytic Residues on the Relative Stabilities of Some of the Intermediates and Transition States in the Citrate Synthase Reaction†

Linda C. Kurz,*,‡ Tanuj Nakra,‡ Richard Stein,‡ Wanda Plungkhen,‡ Marian Riley,‡ Fong Hsu,§ and George R. Drysdale‡

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, Washington University, St. Louis, Missouri 63130

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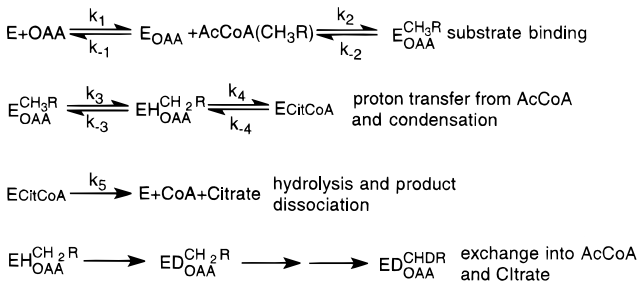
ABSTRACT: This work reports the relative importance of the interactions provided by three catalytic residues to individual steps in the mechanism of citrate synthase. When the side chains of any of the residues (H320, D375, and H274) are mutated, the data indicate that they are involved in the stabilization of one or more of the transition/intermediate states in the multistep citrate synthase reaction. H320 forms a hydrogen bond with the carbonyl of oxaloacetate and the alcohols of the citryl-coenzyme A and citrate products. Enzymes substituted at H320 (Q, G, N, and R) have reaction profiles for which the condensation reaction is cleanly rate determining. None of these mutants can activate the carbonyl of oxaloacetate by polarization. All these mutants catalyze the necessary proton transfer from the methyl group of acetyl-coenzyme A only poorly, a process which occurs in a structurally separate site. Furthermore, all H320 mutants hydrolyze the citryl-coenzyme A intermediate significantly more slowly than does the wild-type. D375 is the base removing the proton of acetyl-coenzyme A. D375E and D375G have greatly diminished ability to catalyze proton transfer from acetyl-CoA. The D375 mutants polarize the oxaloacetate carbonyl as well as wild-type. For D375E, the hydrolysis of citryl-CoA is rate determining. D375G, having no side chain capable of acid−base chemistry in either the condensation or hydrolysis reactions is nearly completely devoid of activity in any of the reactions catalyzed by the wild-type. H274 hydrogen bonds to the carbonyl of acetyl-coenzyme A but also forms the back wall of the oxaloacetate-binding site. H274G cannot properly activate either oxaloacetate or acetyl-coenzyme A, and the condensation reaction is overwhelmingly rate determining. Nonetheless, hydrolysis of the intermediate is impaired. All the enzymes except H320R and H274G show kinetic cooperativity with CitCoA as substrate, indicating changes in the subunit interactions with these latter two mutants. The energetics of citrate synthase are surprisingly tightly coupled. All changes affect more than one step in the catalytic cycle. Within the condensation reaction, the intermediate of proton transfer must occupy a shallow well between transition states close in free energy so that perturbations of one have substantial effects on that of the other.

Citrate synthase (CS)[1] catalyzes a Claisen condensation between the carbonyl of oxaloacetate (OAA)[1] and the acetyl methyl of the thioester, acetyl-coenzyme A (AcCoA).[1] The product of the condensation, *S*-citryl-CoA (CitCoA),[1] remains tightly bound to the enzyme and is efficiently hydrolyzed by the enzyme in a separate step to the final products, CoA and citrate. The working hypothesis for the kinetic mechanism is shown in Scheme 1. X-ray structures have been obtained for two major conformation forms of the enzyme

Scheme 1



(*8*, *9*, *10*). The open form, with its active site fully exposed to bulk solvent, is believed to be a substrate- and product-binding form. The closed form, with its active site buried deep within the protein, is believed to carry out both the condensation and hydrolysis steps of the catalytic cycle. The enzyme is a homodimer with each subunit having two domains. Relative movement of the domains is involved in the open to closed conformation change. The active site contains functional residues from both subunits, the large domain of one subunit and the small domain of the other.

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* Author to whom correspondence should be addressed.

‡ Washington University School of Medicine.

§ Washington University.

[1] Abbreviations: AcCoA, acetyl-coenzyme A; CD, circular dichroism; CitCoA, *S*-citryl-coenzyme A; CS, citrate synthase; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); OAA, oxaloacetate; CMCoA, carboxymethyl-coenzyme A; CMX, carboxymethyldethia-coenzyme A; dethiaAcCoA, dethiaacetyl-coenzyme A; cMDH, cytoplasmic malate dehydrogenase; TIM triosephosphate isomerase.

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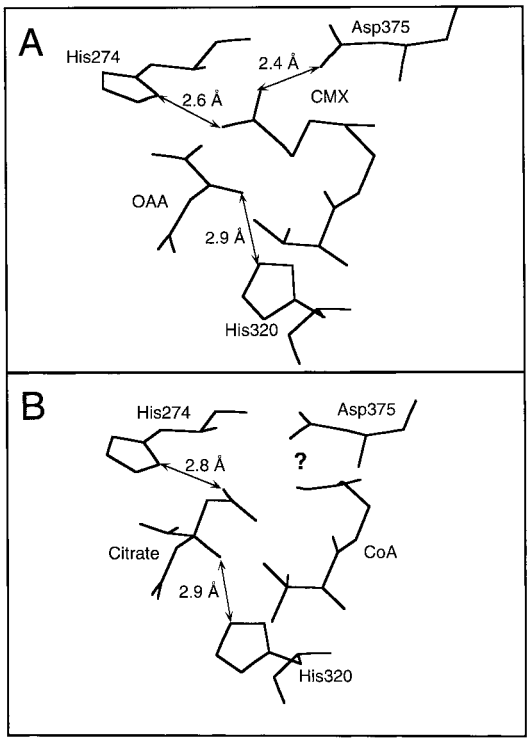

FIGURE 1: (A) Active site of citrate synthase from the structure of the CMX and OAA complex (*3*). (B) Active site of citrate synthase from the structure of the CoA, citrate complex (*8*).

All the chemical steps of the catalytic cycle appear to occur in the absence of bulk solvent. In this work, changes have been made in three active-site residues, which are conserved in all known citrate synthases (*11*): H320, D375, and H274. The interactions of these residues with OAA (substrate) and CMX [an enol(ate) analog] are shown in Figure 1A (*3*), and those with citrate and CoA (products) are shown in Figure 1B (*8*). From the structural studies (ref *5* and references therein), these three residues are representatives of three classes. H320 is a residue of the OAA-binding site, forming a hydrogen bond with the carbonyl of OAA (*6*, *9*) and the alcohols of the CitCoA intermediate (*8*) and citrate product (*8*). D375 is a residue of the AcCoA-binding site and the base removing the proton from the methyl group of AcCoA leading to the formation of the enol(ate) intermediate (*3*, *6*, *12*). Structural studies (*8*) also suggest a role for D375 in the hydrolysis of CitCoA. Partial reaction of CoA-SH and D375 to form aspartyl-CoA might occur in crystals; the unexplained electron density which lead Remington to this conclusion is indicated by a question symbol in Figure 1B. H274 is classified as a residue of both substrate sites. It forms a hydrogen bond with the thioester carbonyl of AcCoA (and presumably that of CitCoA) and with the AcCoA-derived carboxylate of citrate after hydrolysis of the thioester intermediate (*8*). Space-filling structures reveal that this residue is in contact with the OAA methylene and forms the back wall of the OAA-binding site (*6*). H274 is also a "hinge" residue; undergoing large changes in backbone conformational angles during the course of the conformational transition from open to closed form (*11*). In this work, we report results from several physical and kinetic studies which allow a better definition of the roles of these residues and which point the way to further experiments. It has been possible to assign a greater importance of the interactions of particular residues to particular steps in the mechanism. However, the general picture is that the energetics of the active site of citrate synthase are tightly coupled; no amino acid functional group change was found to effect only one part of the catalytic cycle.

MATERIALS AND METHODS

Enzymes.[2] Crystalline citrate synthase from pig heart was obtained from Sigma Chemical Co., St. Louis, MO. Cloned enzymes were prepared as described in ref *13*.

Substrates and Inhibitors. [2-^{13}C]OAA was prepared as described in ref *2*, CMCoA and [1-^{13}C]CMCoA according to ref *14* with modifications as described previously (*2*), and CitCoA as in ref *15*. For experiments which required CitCoA in D_2O, the final G-10 desalting column was run in 1 mM DCl.

Kinetic Methods and Substrate Concentrations. Absorbance data were collected using a Cary 3 spectrophotometer at 20 ± 0.1 °C. Concentrations of AcCoA, OAA, and CitCoA were measured enzymatically using CS. Citrate synthase activity (*16*) 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 [ϵ = 14.1 mM^{-1} (*17*)] or by AcCoA-OAA disappearance which was monitored at 233 nm [$\Delta\epsilon$ = 5.4 mM^{-1} (*18*)].

With CitCoA as substrate, three different assays have been employed as indicated in the description of individual experiments. The concentrations of thioesters (AcCoA/CitCoA) are monitored directly through the characteristic absorption at 236 nm [$\Delta\epsilon$ = −4.96 mM^{-1} (*15*)]. CoA produced from hydrolysis is monitored at 412 nm ($\Delta\epsilon$ = 14.1 mM^{-1}) through reaction of its sulfhydryl with the thiol reagent, DTNB (*17*). OAA produced from cleavage is reduced to malate by cMDH with concomitant oxidation of NADH ($\Delta\epsilon$ = −6.22 mM^{-1}).

In experiments with a transient phase, progress curves with CitCoA as substrate were collected either using a rapid kinetics spectrometer accessory (model RX 1000, Applied Photophysics) for the Cary 3 spectrophotometer or an Applied Photophysics stopped-flow spectrophotometer (model SFMV12) using a 1 cm cell thermostated at 20 °C.

Circular Dichroism. Spectra and titration data were collected and analyzed as previously described (*2*).

^{13}C NMR. ^{13}C spectra at 10 °C were obtained at 150.7 MHz using a Varian Unity 600 spectrometer or at 125.7 MHz using a Varian VXR-500 spectrometer both equipped with a 5 mm multinuclear probe. Proton-decoupled spectra were obtained using Waltz decoupling. The sample buffer was 50 mM Tris-Cl and 1 mM EDTA, pH 7.50, and included 25% D_2O (for internal lock) and 0.15 M acetonitrile (as internal chemical shift standard). Other details were as described previously (*2*).

Proton NMR. 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 fre-

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

quency) during the delay between acquisitions. Spectra were collected at 25 °C with a 90° pulse, and the delay between acquisitions was set to at least five times the T_1 value of the slowest relaxing proton of interest. A few spectra were collected at 600 MHz to improve quantitation of isotope shifted resonances in solvent-exchange experiments.

Solvent Exchange of the Methyl Protons of AcCoA. Enzyme samples for all exchange experiments were exchanged into the buffer for the experiment, 50 mM K[PO_4], pD 7.9 in 99.9% D_2O (low paramagnetic content, Cambridge Isotopes), by a minimum of five cycles of concentration ($\leq$0.5 mL) and redilution ($\sim$3 mL) using centricon-30 (Amicon) centrifugal concentrators, which were exhaustively prerinsed to remove the glycerol storage solution.

For enzymes with relatively rapid exchange kinetics, the fraction of AcCoA methyl protons exchanged was calculated from the ratio of the integrated areas of the acetyl-methyl resonance at 2.35 ppm to the pantothenate methyl resonance at 0.85 ppm. This ratio was corrected for the AcCoA protons lost because of reaction. Oxygen was excluded and the solutions were argon saturated to improve accuracy of CoA determinations. Assays for loss of AcCoA were conducted on the same samples to provide an independent measure of the extent of reaction and agreement was 5−7%. For enzymes with low levels of exchange compared to the reaction rate (WT, H274G, H320Q), improved accuracy was obtained by measuring the appearance with time of the isotope shifted methyl peak containing one deuterium (a triplet since $I = 1$ for D). Extents of reaction were analyzed as before.

AcCoA (Sigma Co., St. Louis, MO) was dissolved in a solution containing 10 mM OAA in 50 mM K[PO_4], pD 7.9. After the AcCoA was assayed enzymatically (final concentration close to 7 mM), enzyme was added and the total volume adjusted with buffer. The solution remained under argon gas at all times. At each time point, an aliquot was removed from the reaction tube, rapidly diluted 7-fold with buffer, and immediately centrifuged in a prerinsed centricon-30 to separate enzyme from the sample. The filtrate was assayed for CoA and AcCoA and the rest of the sample was immediately frozen in liquid nitrogen for later NMR analysis. The extents of reaction and exchange were kept below 20% to minimize the effects of multiple exchanging sites. Data were analyzed as plots of fraction exchange vs fraction reaction. This is equivalent to measuring the ratio of the exchange *rate* to the reaction *rate*.

Solvent Exchange into the Methylene of Citrate with AcCoA and CitCoA as Substrates. The exchange of solvent deuterons into the methylene of citrate was done as in the previous exchange experiments (*19*) (which studied proton exchange into citrate from deuterated AcCoA). For low extents of exchange, we avoid the need for correction for isotope effects and obtain a more direct comparison with data obtained from exchanges starting with CitCoA as substrate (CitCoA was not conveniently deuterated). The OAA was preexchanged before use in the citrate synthase exchange experiments. OAA (solid, free acid, Sigma) was dissolved in 0.1 M DCl (final OAA concentration of 10 mM) and allowed to stand at room temperature 24−30 h, lyophilized, and stored desiccated in the freezer. Before use, the OAA, dissolved in 50 mM Tris DCl buffer, pD 8.9 (final pD close to 7.9), was allowed to stand at room temperature for an additional 2−3 h and then assayed to determine the correct amount to add to experiments.

The AcCoA exchange reactions (total volume of 6 mL) were run at room temperature in 20 mM Tris DCl, pD 7.9. OAA, AcCoA, and DTNB concentrations were 150, 100, and 250 μM, respectively. CS concentrations were varied to allow the reactions to run to completion in 20−40 min.

Exchanges of solvent protons unto the methylene of citrate with CitCoA as a substrate were measured under two conditions. In all cases, the overall reaction, net citrate production, was made irreversible by including DTNB in the reaction mixture. The first condition has only DTNB (250 μM) trapping so that any OAA released after CitCoA (75 μM) cleavage also eventually ends up as citrate. The second type of experiment includes cMDH (2 μM) and NADH (115 μM) to capture and reduce any OAA released after cleavage of CitCoA.

Upon reaction completion as indicated by 412 nm absorbance, the solution was shaken with an equal volume of cold chloroform to denature the protein and stop any further reaction. The layers were separated and clarified by centrifugation. The top yellow aqueous layer was applied to a column containing 2.5 mL of AG 1-X8 (Cl^- form) anion-exchange resin (Bio-Rad) followed by 20 mL of water. The column was rinsed with 20 mL of 10 mM HCl, and finally citrate was eluted with 50 mM HCl. The first 9 mL of eluent was collected, lyophilized, and redissolved in 150 μL of water. Citrate concentrations were measured using a citric acid kit (Boehringer Mannheim) based on detection of the conversion of NADH to NAD^+ in the presence of citrate lyase and cMDH.

Mass Spectrometry of Citrate Samples. For isotope ratio determination by mass spectrometry, the aqueous samples were dried under a stream of nitrogen and the carboxyls esterified by incubation at 65 °C for >5 h with 50 μL of 2.5 M HBr in *n*-propanol. An aliquot of the derivatization reaction solution (2 μL) was mixed with glycerol (1 μL) for spectrometric analysis. Fast atom bombardment (FAB) analyses were performed by a VG ZAB−SE double-focusing mass spectrometer coupled to an Opus 3.2 data system. An Ion Tech FAB gun was operated at 8 kV (1 mA) with Xe as the bombardment gas. For each analysis, 12 scans (from 250 to 400 Da) were collected and signal averaged. The positive-ion FAB mass spectrum of tris-*n*-propyl citrate gives a major MH^+ at 319 which exhibits a >50-fold increase in sensitivity in comparison to the negative ion FAB mass spectrum of underivatized citric acid. After correcting for natural abundance, the deuteration of the citric acid can be easily measured from integration of the areas of the deuterated citric acid *n*-propyl esters (d_2, d_3, d_4 starting from d_2-OAA; d_0, d_1, d_2, d_3, d_4 starting from d_0-CitCoA). A sample of authentic d_4-citric acid (C/D/N Isotopes, Inc.) showed no loss of deuterium when subjected to this procedure.

RESULTS

Kinetic Constants and Inhibitor Dissociation Constants. Table 1 compares kinetic constants for the mutants with substrates AcCoA and OAA along with ternary complex dissociation constants for inhibitors, dethiaAcCoA and CMCoA, from CS-OAA. Direct measurements of dissociation constants are needed in addition to kinetic constants

Table 1: Kinetic Constants (OAA and AcCoA) and Inhibitor Dissociation Constants (dethiaAcCoA and CMCoA) for Mutants in Three Catalytic Residues of Citrate Synthase

enzyme	K_m^{OAA} (μM)	K_m^{AcCoA} (μM)	k_{cat} (min^{-1})	$K_d^{dethiaAcCoA}$ (μM)	K_d^{CMCoA} (μM)
WT	5.9[a]	5.1[a]	7500	2.3[b]	0.02[c]
			OAA Site		
H320Q	85	126	37	14[b]	0.6
H320G	43[a]	89[a]	16[a]	9[b]	0.2
H320N	38	27	2.2	10[b]	0.03
H320R[h]	8	5	0.03		17
			AcCoA Site		
D375G[e]			no reaction	$\leq$0.01[b]	$\leq$0.003[d]
D375E	0.5	0.7	14	0.5[b]	1.9[d]
			OAA/AcCoA Site		
H274G	3.5[f]	5.4[f]	1[g]	2[b]	9.2[d]

[a] Ref *15*. [b] Ref *13*. [c] Ref *2*. [d] Ref *1*. [e] We failed to find convincing citrate synthase activity for this mutant. [f] Ref *41*. [g] Our preparations of this mutant have less activity than reported in ref *41*. [h] The low activity of H320R required use of high protein concentrations which results in an unacceptably high blank rate as a consequence of DTNB chemical modification of the protein. Thioester disappearance at 233 nm was used to measure activity.

because of the possibility that the latter are complex functions of the rate constants for elementary steps. The ground-state analogue of AcCoA, dethiaAcCoA, can undergo enzyme-catalyzed exchange of the protons of its methyl group (*13*). CMCoA is a tight-binding analogue of the enol(ate) intermediate of AcCoA.

Near-UV (250−300 nm) CD Spectra of Free Enzymes, Binary Complexes with OAA, and Ternary Complexes with CMCoA. The near-UV CD spectra of proteins arise from induced CD in the chromophores of the aromatic amino acids. The specific positions of chromophore side chains relative to the chiral peptide backbone are the origins of these signals, and changes in them are sensitive monitors of conformation. A similar induced CD is frequently observed for complexes of enzymes with ligands whose chromophores absorb in this region and may be similarly interpreted.

For CS and its mutants, the spectra of the free enzymes show no significant differences (data not shown).[3] Differences between the enzymes become apparent in the spectra of binary and ternary complexes. The near-UV CD spectra of free WT CS as well as its complex with OAA are shown in Figure 2A. Note the increased intensities of the CD spectrum of the WT-OAA complex below 290 nm. Panel A also shows *the difference spectra* (CS-OAA minus CS) for WT as well as the active-site mutants studied in this work. Since the keto form of OAA has no chromophore in this spectral region, the *changes in the CD spectrum of the enzyme which accompany OAA binding are a result of a conformation change in the enzyme*. Only H274G responds to the binding of OAA like WT, and the small change for D375E may not be beyond experimental error. The CS-OAA minus CS difference spectra for all H320X is close to zero; no H320 mutant accomplishes the conformation change detected by this probe.

The CS-OAA−CMCoA spectra (Figure 2B) for H320G, N, and Q are identical to WT. These large CD changes in ternary complex spectra are characteristic of the closed form of the enzyme (*2*). The ternary complex spectra of D375E and H274G have a slightly smaller extent. These differences

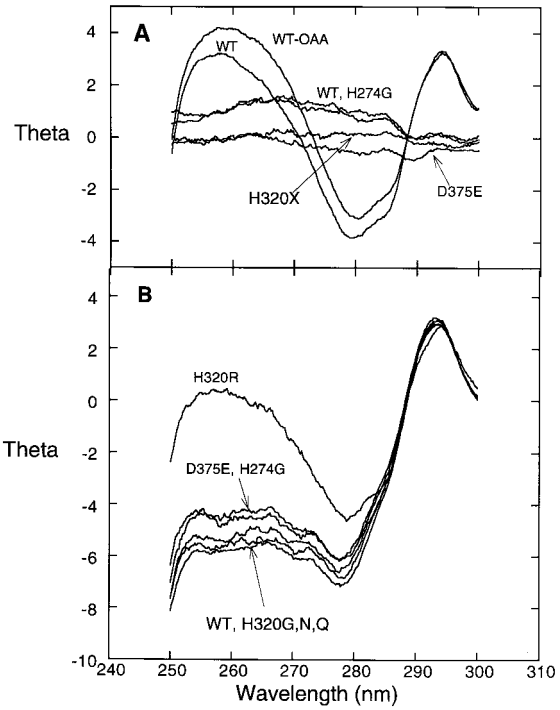

FIGURE 2: Near-UV CD spectra of CS, binary CS-OAA and ternary CS-OAA−CMCoA complexes. (A) Upper two traces are WT-OAA and WT (spectrum of enzyme with no ligand present). Lower traces are difference spectra (CS-OAA minus CS) for WT and the mutants. (B) Spectra of ternary CS-OAA−CMCoA complexes. Conditions are as described previously (*2, 13*).

are less than those observed between ternary complexes of WT with various CoA analogues (*13*). Yet, X-ray structures of the ligands in these WT−CoA analogue complexes (*3, 8, 9*) are very similar. Therefore, we believe the small differences between D375E, H274G, and WT we see here are not structurally significant.

The ternary complex of H320R resembles binary complexes of CS with CoA analogues rather than ternary complexes (*1*). It is the only mutant so far found to behave in this manner.

NMR Probes of OAA and AcCoA Activations. Table 2 gives the chemical shift information for [2-^{13}C]OAA and [1-^{13}C]CMCoA (enol(ate) analog) bound to CS binary and ternary complexes; chemical exchange regime (free CMCoA

[3] We were unable to study "free" D375G. Preparations of this mutant enzyme contain varying amounts of tightly bound ligands which could not be removed without some irreversible denaturation with consequent effects on the spectra.

Table 2: Chemical Shifts and Exchange Regime (Ternary Complexes) of [2-^{13}C]OAA and [1-^{13}C]CMCoA for Complexes of Three Catalytic Mutants of Citrate Synthase

enzyme	CS-OAA* (ppm)	CS-OAA*-CMCoA (ppm)	CS-CMCoA* (ppm)	CS-OAA-CMCoA* (ppm)
none	199.8[a]		178.1(−COO$^-$), 174.9(−COOH)[b]	
WT	204.4[a]	206.6[a] (slow exchange)	177.4[b]	175.2[b] (slow exchange)
		OAA Site		
H320Q	201.0	202.9 (slow exchange)	177.3	175.6 (slow exchange)
H320G[c]	200.8	202.3 (slow exchange)	176.1	176.1 (slow exchange)
H320N	201.8	202.6 (slow exchange)	177.7	175.4 (slow exchange)
H320R	200.8	200.8 (slow exchange)	177.1	177.1 (not determined)
		AcCoA Site		
D375G[d]	203.9	206.1 (slow exchange)	177.9	171.6 (slow exchange)
D375E[d]	203.0	205.8 (slow exchange)	177.7	173.1 (slow exchange)
		OAA/AcCoA Site		
H274G[d]	203.0/202.4	203.1 (slow exchange)	177.5	176.4 (slow exchange)

[a] Ref *20*. [b] Ref *2*. [c] Ref *15*. [d] Ref *1*.

ligand exchanging with the complex) is given for ternary complexes.

The chemical shift of a nucleus is sensitive to its electrical and magnetic environment. Thus, resonance position can provide useful information on charge distribution and protonation state, important factors for understanding mechanism which are frequently not easily deducible even from high-resolution crystal structures. The line width (T_2) or chemical exchange regime of resonances is sensitive to dynamic processes and can provide information on dissociation rates, environmental fluctuations, and conformation changes.

Polarization of the carbonyl carbon of OAA is a major catalytic strategy of citrate synthase. By increasing the positive charge at the reaction center, its reactivity is increased for condensation with the nucleophile derived from AcCoA. Two experimental techniques have been used to detect carbonyl polarization in CS: the large deshielding (6−7 ppm; see WT values in Table 2) of the carbonyl carbon chemical shift found in NMR spectra of ternary complexes of [2-^{13}C]OAA with AcCoA analogues, particularly the enol(ate) analogue, CMCoA (*1*, *2*, *20*), and the significantly decreased OAA C═O bond order reflected in the lowered carbonyl stretching frequency found in the IR spectra of those same complexes (*21*).

For the WT, the CMCoA carboxyl chemical shift change (*1*) from that in free solution is now attributed to unusual geometry in the hydrogen bonding of the inhibitor carboxyl with residues in the active site, particularly the one to D375 rather than to a change in the protonation state of the inhibitor (*4*).[4] Even in the case of D375G-OAA-*CMCoA with its extremely low isotropic chemical shift (shielded by over 6 ppm compared to the anion in solution), solid-state NMR experiments indicate an anionic carboxylate probably bonded to a protonated H274 (*4*). Exactly what hydrogen bond geometry changes underlie the changes in the carboxylate chemical shift (some of which are quite large) in complexes with the mutant enzymes will have to await X-ray structures and possibly further solid-state NMR studies. What is clear is that unusual forces and geometries are present in this part of the active site, although how they are brought to bear on catalysis is still obscure.

CS-Catalyzed Hydrolysis of CitCoA: Partitioning of the Intermediate. Formation of the chemical intermediate (CitCoA) almost certainly marks the point at which the enzyme switches from ligase to hydrolase. Although the presence of kinetic cooperativity complicates a complete analysis, it has generally been possible to measure the difference in heights of the free energy barriers between the forward hydrolysis and the reverse condensation (cleavage) steps using the relative initial rates at which CitCoA is cleaved back to reactants (OAA/AcCoA) or proceeds to products (Citrate/CoA), v_i^{OAA}/v_i^{CoA}.

For the WT, the initial ratio of the rate of CitCoA cleavage to hydrolysis, 0.4 (*22*, *23*), indicates that hydrolysis is somewhat favored over cleavage. This initial partitioning of the chemical intermediate is profoundly changed in the mutants except for H274G and ranges from ∼0 to 8 (Table 3).

Figure 3A (inset) shows the partitioning experiment for H274G. Curves a and b show the time courses for the production of OAA and CoA, respectively. The rate of CitCoA hydrolysis to CoA/citrate is substantial (k_{cat} = 150 min^{-1}) and is faster than the steady-state rate of CitCoA hydrolysis for WT at the same protein concentration. However, the k_{cat} sustained by this mutant with the natural substrates, AcCoA/OAA, is very low (1 min^{-1}, Table 1). Thus, CitCoA is rapidly processed by this mutant. However, the CitCoA which cleaves to AcCoA/OAA funnels back through the enzyme to CoA/citrate only very slowly. We have demonstrated this in two ways as shown in Figure 3A, inset. First, a residual slow rate of CoA production is detected after the bulk of the CitCoA has been processed.

[4] The sign and magnitude of the change in the chemical shift of the carboxyl ^{13}C resonance when the ternary (but not the binary) complex form is consistent with protonation of the inhibitor's carboxyl (*1*, *2*). Protonation of the intermediate analogue inhibitor was supported by structural and solution evidence. As shown through the pH dependence of the equilibrium constant, proton uptake occurs on a macroscopic level when the ternary complex forms. The isoelectric point (and thus the overall charge) of the complex with CMCoA is the same as that with a neutral ligand (such as acetonyl-CoA). There is a very short hydrogen bond between the oxygen of the carboxyl of the active-site base, D375, and the oxygen of the inhibitor with a heteroatom to heteroatom distance of 2.4 Å (*3*). Certainly a proton is present here. We concluded that protonation implied a neutral enol form of AcCoA as the reactive intermediate in the citrate synthase reaction. Despite this extensive circumstantial evidence, the results of recent solid-state NMR experiments (*4*) are not consistent with simple protonation but instead suggest a negatively charged CMCoA carboxylate participating in atypical hydrogen bonds with the protonated active-site base, D375, and with neutral H274. So present evidence favors an enolate intermediate with unusual hydrogen bonding, the significance of which is yet to be determined.

Table 3: Properties of CitCoA Progress Curves and Partitioning of the Chemical Intermediate to Reactants and Products

enzyme	progress curve type	presence of transient?	kinetic constants (k_{cat} min^{-1}, K_m μM)	V_i^{OAA}/V_i^{CoA}
WT	three phases	yes[b]	*a*	0.4[b]
		OAA SITE		
H320Q	accelerating[a]	yes[b]	*a*	≤0.03
H320G	accelerating[a]	yes[b]	*a*	≤0.03
H320N	accelerating[a]	yes[b]	*a*	≤0.03
H320R	normal	yes[b]	0.4, 1.5	≤0.03
		AcCoA Site		
D375G	no reaction[d]			
D375E	three phases	yes[b]	*a*	8
		OAA/AcCoA Site		
H274G	normal	no	150, too tight to measure	0.2

[a] Cooperativity according to either Monod or Koshland models—see ref *15* and Figure 3. [b] See discussion in text. For H320N, the burst rate at 30 μM CitCoA is 3000 min^{-1}, greatly exceeding the value of k_{cat}. [c] Ref *23* and confirmed by the present workers. [d] No hydrolysis rate beyond the nonenzymatic one could be demonstrated with this mutant.

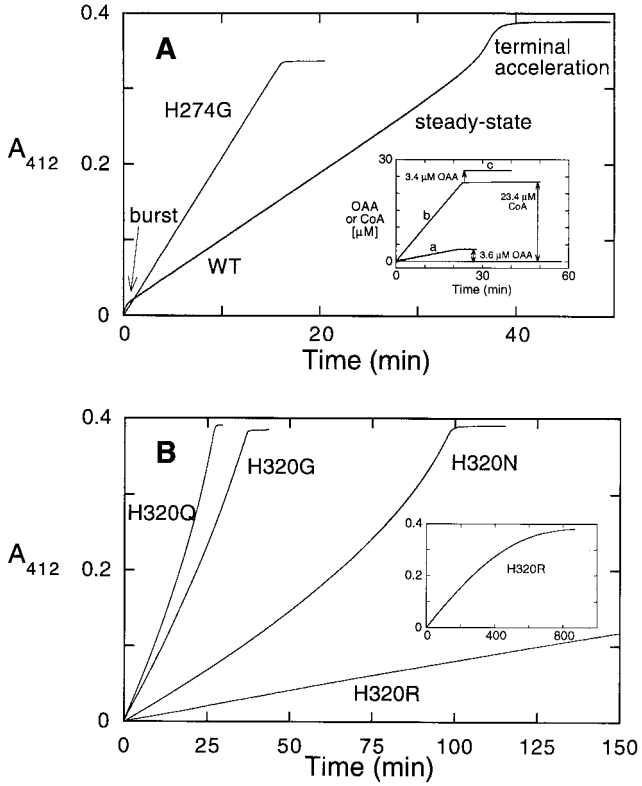

FIGURE 3: Progress curves for CS-catalyzed hydrolysis of citryl-CoA. (A) WT and H274G−CoA detected by DTNB (27 μM CitCoA, 21 nM WT, and H274G). (Inset) Partitioning of the CitCoA intermediate by H274G. Trace a: Production of OAA from 30 μM CitCoA, OAA release irreversible. Trace b: Production of CoA from 30 μM CitCoA, only CoA release irreversible. Trace c: Production of OAA from CitCoA after CoA production had nearly ceased. At the time indicated by the arrow; approximately 23 min after the reaction had been initiated, cMDH and NADH were added to detect OAA still present after CoA production had slowed to nearly undetectable levels. (B) H320X−CoA detected by DTNB (27 μM CitCoA 17 nM H320X). (Inset) Full-time course for H320R.

Second, the fate of the "missing" CitCoA can be determined by adding NADH/cMDH at the point at which CoA production has virtually ceased. The OAA which has formed by the cleavage of CitCoA is then rapidly reduced by cMDH, and the NADH oxidized quantitatively accounts for the rest of the CitCoA initially present in the reaction mixture (Figure 3A, inset, line c).

Some quantitative comparisons between mutants and WT can be made in terms of the rate constants of Scheme 1. The scheme omits steps related to conformation changes as well as several possibly reversible steps during CitCoA hydrolysis (which we have represented by the single, irreversible step, k_5). These simplifications do not effect the present analysis. In terms of the rate constants of Scheme 1, k_{cat} is given by eq 1. We have grouped together some of these terms to emphasize their physical significance. The first term in the numerator and denominator of eq 1, *f*, is the apparent rate constant for the forward step of the condensation reaction, and the second term, *r*, is the apparent rate constant for the reverse step of the condensation. Similar terms appear in the partition ratio.

$$k_{cat} = \frac{\dfrac{k_3k_4}{k_4+k_3+k_{-3}}k_5}{\dfrac{k_3k_4}{k_4+k_3+k_{-3}} + \dfrac{k_{-4}(k_3+k_{-3})}{k_4+k_3+k_{-3}} + k_5} = \frac{fk_5}{f+r+k_5} \quad (1)$$

The partition ratio, v_i^{OAA}/v_i^{CoA} for CitCoA as substrate, is given by eq 2.

$$\frac{v_i^{OAA}}{v_i^{CoA}} = \frac{\dfrac{k_{-4}(k_3+k_{-3})}{k_4+k_3+k_{-3}}k_{-2}}{k_5\left(k_{-2} + \dfrac{k_3k_4}{k_4+k_3+k_{-3}}\right)} = \frac{k_{-2}r}{k_5(k_{-2}+f)} \quad (2)$$

CS-Catalyzed Hydrolysis of CitCoA: Progress Curves. With CitCoA as substrate, the progress curves for the disappearances of CitCoA reactant and the appearances of CitCoA and OAA products are compared for the WT and mutant enzymes in Figures 3 and 4 and Table 3. The kinetic behavior is rich and varied. At the semiquantitative level, this is understandable by changes in the relative free energies of the intermediates and transition states along the internal reaction coordinate as modulated by the kinetic cooperativity originating in intersubunit interactions. The relative magnitude of the amplitudes, the rates of the phases, and the predominant products differ from one mutant to another.

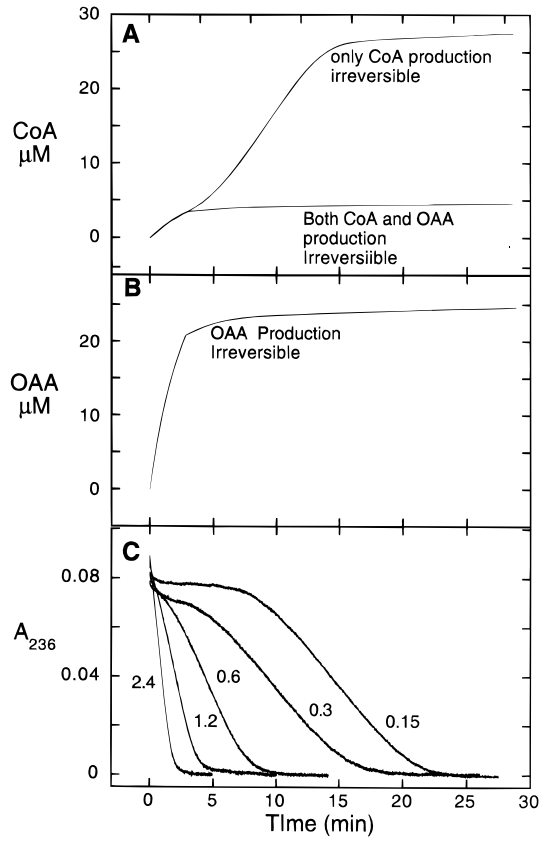

FIGURE 4: Progress curves for CitCoA as a substrate of D375E. (A) Upper trace is CoA production from 28 μM CitCoA detected with DTNB which also renders CoA release and the overall reaction irreversible. Lower trace is CoA production form 28 μM CitCoA in which the final steps of both the reverse of condensation and hydrolysis are made irreversible by trapping the respective products upon release from the enzyme. (B) OAA produced from 28 μM CitCoA trapped by the cMDH/NADH system. (C) Disappearance of thioester absorbance at 236 nM at a series of D375E concentration from 0.15 to 2.4 μM as indicated on the graph; CitCoA concentration is 30 μM.

For the WT (*23*−*27*), the reversal and forward reactions occur simultaneously during the burst and steady-state phases with the forward reaction predominating. The terminal acceleration phase represents product formation from OAA and AcCoA accumulated during the first two phases. The rate in the steady-state phase is very much less than the maximal rate obtained when reactions are started with AcCoA/OAA.

OAA Site: H320Q, G, N, and R. Three of the H320 mutants, H320Q, H320G, and H320N, have accelerating progress curves (Figure 3, panel B). The CitCoA concentration dependencies (the profound substrate inhibition with a low but nonzero rate at high CitCoA concentration) and the lack of dependence of the progress curve shape on the enzyme concentration characterize all these mutants. See ref *15* for extensive discussion of H320G; similar data for H320Q and H320N are not shown here. All these mutants of H320 have rates for CitCoA hydrolysis *larger* than the rates starting the reaction from AcCoA/OAA in contrast to the case of WT. Interestingly, the CitCoA hydrolysis rates follow the same order as the overall k_{cats}, H320Q > H320G ≫ H320N ≫ H320R as shown by the progress curves (Figure 3, Panel B), which were all collected at the same enzyme concentration. Unlike WT and D375E, H320Q, G, and N do not exhibit a final separate acceleration phase since they do not partition CitCoA back to OAA/AcCoA, vide supra.

The progress curve shape for H320R (Figure 3, panel B, inset) appears to be normal as is the dependence of the rate on CitCoA; *this enzyme shows no kinetic cooperativity (nor does H274G).* The k_{cat} for production of CoA/citrate from CitCoA greatly exceeds the k_{cat} for the reaction starting with the normal substrates, AcCoA/OAA for both H320R and H274G. However, the k_{cat}s for hydrolysis are still quite low compared to the overall k_{cat} of the WT with the normal substrates.

AcCoA Site: D375E and G. As in WT (Figure 3A), there are also three phases for D375E (Figure 4A), but the data show (Figure 4, panels A and B) that the hydrolysis is interfered with much more than the cleavage with the result that the predominant products of the burst and steady-state phases are OAA and AcCoA rather than CoA and citrate as in the WT. Like WT, the steady-state rate is low compared to the rate if the reaction had been started with the normal substrates, OAA and AcCoA. As would be expected then, the terminal acceleration phase is very large as it represents product formation as the inhibitory CitCoA is used up. As in the case of WT, if the enzyme concentration is raised high enough, all the reaction occurs in the burst phase.

D375G does not react at all.

OAA/AcCoA Site: H274G. H274G shows no burst and has only two phases for the utilization of CitCoA. The major phase of the progress curve represents primarily production of CoA and citrate. It is zero order (linear) over all practical substrate concentrations (Figure 3A, inset; Figure 4A); the K_m must be very much less than 1 μM. The apparent missing extent (panel A, Figure 3) is a consequence of the *very slow* feed through of the OAA/AcCoA produced during the steady-state of CitCoA utilization. As with WT and D375E, if OAA produced by CitCoA cleavage is trapped by cMDH/NADH, no final phase occurs.

CS-Catalyzed Exchange of Solvent Protons into Substrate AcCoA and Product Citrate. Our working hypothesis assumes that condensation takes place in two separate steps. In the first step, a proton transfer occurs to the active-site base, D375, generating an unstable enol(ate) intermediate from AcCoA. In the second step, the actual condensation takes place to generate CitCoA. [For the analogous reaction catalyzed by malate synthase, evidence been presented for this stepwise nature of the process (*28*).] Exchange of solvent protons into either the methyl of the AcCoA substrate pool or the methylene of the citrate product requires that the hydrogen initially removed from the substrate exchanges with those from the bulk solution (first step of last line of Scheme 1) before undergoing an irreversible step. Once exchange has occurred, subsequent removal/return of that deuterium is subject to a primary isotope effect. A further complication arises from the existence of three exchanging sites; several cycles of proton transfer and exchange could occur before further reaction to product or dissociation into the substrate pool. In the general case, it is not possible to write an explicit expression relating the rate and extent of the exchanges to the elementary rate constants of the individual steps. Without simplifying assumptions, it is also not possible to relate these data to the average number of "reversals" of the proton-transfer event occurring at each

Table 4: Solvent Exchange Rates and Extents for Citrate Synthase

enzyme	exchange rate relative to reaction rate starting with AcCoA and OAA	deuterium excess in citrate: starting with AcCoA and d_2OAA	deuterium excess in citrate: starting with d_0-CitCoA, OAA release, reversible	deuterium excess in citrate: starting with d_0-CitCoA, OAA release, irreversible
WT	none detected	0.36	0.23	0.14
		OAA Site		
H320Q	≤0.05	0.18	0	0
H320G	1.0	0.59	0	0
H320N	1.2	1.4	0	0
		AcCoA Site		
D375E	0.6	1.25	1.5	0.65
D375G	none detected	none detected	none detected	none detected
		OAA/AcCoA Site		
H274G	≤0.1	0.17	slight	0

catalytic cycle (*19*). Despite these complexities, a number of semiquantitative conclusions can be drawn from these studies.

Rate of Exchange into AcCoA. Table 4 records the rate of exchange of solvent deuterons into the methyl group of substrate AcCoA relative to the rate of reaction. Because one of the products is trapped and there is no contribution from reversal of the overall CS reaction, the reversal of proton transfer thus detected occurs before the release of the final product. For the WT, no exchange into free AcCoA can be observed. In this case, the likely reason is that the commitment to catalysis is sufficiently high that the amount of exchanged AcCoA released back into solution compared to that which goes on to citrate is too low to be detectable in the large substrate pool. However, for mutants (all those studied here) which obey prior equilibrium mechanisms, we expect to observe exchange. The very slow turnover rates for all these enzymes are accompanied by slow exchange rates. This was initially an unexpected result for mutations in the OAA site (*13*). If proton transfer precedes condensation and if condensation were the only step deleteriously effected by OAA site mutations, we would have expected rapid AcCoA exchange relative to reaction rate.

The behavior of H320N and H320G contrasts with that of H320Q. For the former two H320 mutants, the rate of exchange is about equal to the rate of reaction, while for H320Q that rate of exchange is very much lower. Note, however, that the K_ms (K_ds) for AcCoA for all these mutants are high, *especially* for H320Q (Table 1). Therefore, the observed exchange rate cannot be controlled by slow dissociation of bound AcCoA for any of the H320 mutants and, in particular, the low rate for H320Q in comparison to that for the other mutants must have some other explanation (see below). Since none of these H320 mutants can cleave CitCoA, the reversal of proton-transfer detected is occurring at the AcCoA activation step and is not a consequence of the reversal of the entire condensation process.

D375E also shows a substantial exchange rate relative to the rate of reaction. Even in the case of this mutant (exchange rate is about 50% the rate of reaction) for which the K_m for AcCoA is quite low (Table 1), the k_{cat} is still sufficiently low that it is unlikely that the rate of exchange observed is limited by the dissociation rate of AcCoA (see section below on exchange into citrate). H274G shows only a low rate of exchange relative to that of reaction.

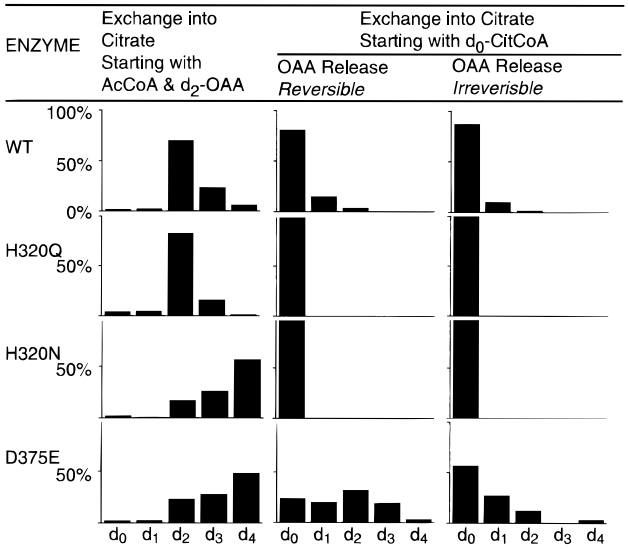

FIGURE 5: Patterns of isotopes obtained from exchange of solvent deuterons into citrate product with AcCoA, OAA, or CitCoA as substrates for WT, H320Q, H320N, and D375E. Isotope distributions were determined by mass spectroscopy as described in the text.

CS-Catalyzed Exchange of Solvent Protons into Product Citrate. Even when solvent exchange of the AcCoA methyl protons may not be detected in substrate AcCoA for whatever reason, exchanged hydrogens will appear in the methylene of citrate. Using mass spectrometry to assess exchange into the methylenes of citrate is sensitive and accurate and allows study of exchange when the initial substrate is the chemical intermediate, CitCoA. The deuterium excesses for the exchange reactions for the mutants and wild-type are recorded in Table 4; a maximum value of 2.0 (expressed as additional deuterium excess in this case) is possible for reactions starting with d_2-OAA/AcCoA. Reactions starting with d_2-OAA and CH_3-AcCoA should contain 100% d_2-citrate if no exchange occurred while complete exchange would have 100% d_4. (If OAA preexchange was complete, no isotope less than d_2 should be present.) Starting with d_0-CitCoA as substrate, it is possible to obtain citrate containing from 0 to 4 deuteriums. OAA released into solution can undergo nonenzymatic exchange to d_1 and d_2-OAA and then subsequently react with activated AcCoA containing from 0 to 2 deuteriums. In Figure 5 are shown the actual patterns of isotopes (corrected for natural abun-

dance) in citrate under the indicated conditions and starting from the indicated substrate, by WT; H320Q and H320N (representing the extremes of the behavior of the H320 mutants) and D375E.

Results of exchange experiments using CitCoA allow an interesting distinction when considered together with the CitCoA partitioning data. If significant amounts of exchange are occurring because the condensation reaction is reversing all the way back to free OAA, then significant amounts of d_3 and d_4 citrate will be obtained because the released OAA can exchange its methylene protons nonenzymatically. If all the exchange is occurring without release of substrates bound to the enzymes, then only d_1- and d_2-citrate will be obtained. This distinction between exchange occurring from reversal of the entire condensation step back to free reactants vs exchange occurring as a consequence of exchange of bound states was not possible in previous experiments (*19*).

Since mutants which cannot cleave CitCoA (i.e., H320) do not exchange its methylene protons during hydrolysis, we conclude that exchange of solvent hydrogens into the methyl of AcCoA or methylene of citrate is possible only during the condensation part of the catalytic cycle. In fact, none of the H320 mutants produce any exchanged citrate starting with CitCoA as substrate (Table 4).

For the WT, exchange is detected in citrate where substantial deuterium excess is found whether the reaction is started from OAA/AcCoA or CitCoA (Table 4 and Figure 5). These results are similar to those reported in ref *19*. Starting with CitCoA, somewhat smaller deuterium excess is observed if the OAA release is made irreversible. These results are most consistent with the exchange occurring in the condensation half-reaction only when the substrates are bound to the enzyme, but most of this may occur from reversal of the entire condensation sequence (while remaining bound to the enzyme, vide infra).

Exchange can logically take place by reversal of the proton-transfer step itself or by reversal back from CitCoA of the entire condensation sequence. It appears reasonable that the lifetime of the stable intermediate, CitCoA, will be longer than that of the unstable intermediate, AcCoA enol(ate). Thus, for enzymes which have a substantial CitCoA cleavage rate, most of the exchange would occur by reversal from CitCoA to AcCoA/OAA since more time will be allowed for the protonated active-site base to access the bulk solution. While not excluding the possibility of exchange by reversal of the proton-transfer step, Myers and Boyer (*19*) also favored exchange by complete reversal of both steps in the condensation reaction for the WT. Simulation of the complete mechanism for exchange confirms that this is likely the case for any enzyme with a significant CitCoA cleavage rate.

However, the results with the H320 mutants show that exchange by reversal of the proton transfer step does occur. CitCoA formation is irreversible for these mutants (Table 3). Yet they show substantial amounts of exchange into the methylene of citrate when the reaction is started from AcCoA/OAA. H320Q shows slightly less exchange than the WT, while H320G shows substantially more and H320N shows close to complete equilibration with the solvent (1.4 equiv of the 2 possible).

DISCUSSION

Effects of Mutations: General Comments. All of the mutations studied have larger effects on k_{cat} than on the K_m; consistent with a *primary role for these residues in the stabilization of one or more of the transition states/activated intermediates within the central complexes (29).* In the discussion which follows we have maintained the structural classification based on whether the residue appears to interact primarily with OAA, AcCoA, or both. However, a major conclusion of this work is that there is no corresponding functional classification. No mutant has been found that affects only a single step in the mechanism. The linkage appears to be especially tight for changes in H320 (OAA site) where every function previously identified is affected; polarization of the OAA carbonyl, proton transfer from AcCoA, and hydrolysis of the CitCoA intermediate. D375 (AcCoA site) is critically important to both hydrolysis of the CitCoA intermediate and to proton transfer from AcCoA (since it is the active base) while seeming to play little role in OAA carbonyl polarization. H274 (OAA/AcCoA site) has the expected importance within the condensation reaction (OAA and AcCoA activations) but also has an unexpectedly large role in the stabilization of the transition states/intermediates in the hydrolysis of the CitCoA intermediate.

OAA Site: H320Q, G, N, and R

Conclusions from Kinetic and Inhibitor Constants. The K_ms for H320Q, G, and N are all greater than those of the WT. This is the pattern expected for a residue with a binding as well as a catalytic role. However, the k_{cat}s for the H320X mutants are negatively correlated with the K_ms for both substrates; the more active mutants bind substrates more loosely. Overall, the interactions provided by H320 imidazole stabilize *both* the ground state and transition state. However, *within* the series Q, G, and N, those residues providing better hydrogen bonding to the OAA carbonyl (i.e., Q, then G plus a water molecule) destabilize the ground state while stabilizing the transition state.

Prior OAA binding increases the affinity of the WT enzyme for AcCoA or its analogues by a factor between 10 and 20 (*1, 13, 30, 31*). For the H320Q, G, and N mutants, the destabilization of the OAA binary complex is propagated to the ternary complex with AcCoA even though there is no direct interaction between AcCoA and H320. The OAA site (H320Q, G, and N) mutants have slightly reduced affinity for *both* AcCoA-site ground and enol(ate) analogues confirming the propagation of effects at the OAA site to both the ground state and transition state for proton transfer.

For H320R, steric and charge issues confuse the interpretation of kinetic constants.

Effects on the CS Conformation Change. The near-UV CD spectra of mutants at H320 fail to respond to OAA binding in the same way as WT, indicating differences in the structures of the binary complexes. This may be a local change not reflecting the overall ability to form the closed conformation.

The "closed" form finally reached by the mutants H320N, Q, and G in ternary complexes is likely very close in structure to that of the WT. There are no significant differences between the ternary complex CD spectra of H320N, Q, G, and WT. All binary complexes of CMCoA which have been

studied are in fast NMR exchange where excess ligand results in a population-weighted chemical shift, *but in all ternary complexes, CMCoA is in slow exchange with the free ligand*; that is, the presence of excess CMCoA has no effect on either the position or line width of the bound resonance. This is true even for H320G where the chemical shift in the ternary complex remains at the binary complex value. Although the K_m (AcCoA) and K_d (ground-state analogue, dethiaAcCoA) values are 4−20-fold larger than those of WT, it appears that the H320 mutants with Q, G, or N substitutions can decrease the dissociation rate constant for the AcCoA intermediate-state analogue, CMCoA. Preliminary SAXS/SANS data (S. J. Henderson and L. C. Kurz, unpublished results) record a reduction of the radius of gyration on formation of the binary OAA and ternary OAA−CMCoA complexes with H320G that are within experimental error of those of WT. *Thus, the ternary complexes of even these very defective mutants possess properties believed to be associated with the closed conformation of the enzyme.* These results do not exclude dynamic differences or differences in the energy cost of achieving that closed form..

The unusual shapes of the CitCoA progress curves observed for WT, D375E, H320Q, G, and N are a manifestation of kinetic cooperativity arising from subunit interactions (*15*). For H320Q, G, and N, the cooperative interactions settle down into a steady state by the collection of the first data point (Figure 3), while for WT and D375E, the transient leading to establishment of the allosteric steady state (burst phase) is still evident for some time.[5] An extensive analysis of the case of H320G has been presented previously (*15*). H320Q and N show very similar behavior. At the root of cooperativity is the occupation of *both active sites at the same time* by the high-affinity chemical intermediate, a situation which will occur only rarely, if ever, during normal turnover. One successful model (*15*) of these data supposes that occupation of both active sites by CitCoA "locks" both subunits into the "closed" form slowing or preventing egress of products and thereby severely hampering turnover.

Since the CD spectrum of H320R-OAA−CMCoA more closely resembles that of binary complexes of CS with CoA analogues (*2*) than spectra of other ternary complexes (*13*), we reasonably conclude that this mutant fails to close. (Steric hindrance from its size or the charge of the R side chain may be responsible.) Note that H320Q, G, and N, all of which can close, also all show kinetic cooperativity with CitCoA while H320R, which we believe cannot reach the closed form, shows no cooperativity.

Effects on OAA Activation (Carbonyl Polarization). None of the mutants of H320 are able to significantly polarize the carbonyl of OAA; the chemical shift of the OAA carbonyl in both binary and ternary complexes of the H320 mutants shows minimal deshielding (2−2.5 ppm) in comparison to the free solution value (Table 2). Even the glutamine substitution fails to promote polarization despite the ability of glutamine to hydrogen bond to the carbonyl in a manner similar to histidine imidazole (S. J. Remington et al., unpublished modeling data). Chemical shift changes of the magnitude actually seen in these mutant complexes are commonly attributed to nonspecific environmental effects, but may represent the portion of the polarization which results from the general positive electrostatic potential in this area of the active site. The active site contains several positively charged residues at least two of which interact directly with the OAA carbonyl. The presence of H320 is required for the major effect even though it appears (*5, 32*) to be uncharged. These results are quite similar to those obtained with H95 substitutions in TIM, where an apparently uncharged H95 performs a similar role in the polarization of the carbonyl of substrate dihydroxyacetone phosphate (*33, 34*). The glutamine substitution of H95 also fails to polarize the substrate carbonyl in that enzyme.

Effects on Ability to Catalyze Proton Transfer from AcCoA. The chemical shifts of the carboxyl of the enolate analogue in ternary complexes of H320Q, G, and N show small to no differences from the ternary complex of WT. This suggests that the final hydrogen-bonding environment experienced by the analogue in these complexes is not greatly perturbed by OAA site changes. However, all these mutants show a profound defect in their ability to activate the ground-state of AcCoA for the essential proton-transfer reaction. While the H320 mutants show either the same or more exchange of solvent protons into AcCoA or citrate methylene compared to WT, the *rate of exchange remains very slow*, comparable to their slow k_{cat}s. Previously reported data on the CS catalyzed exchange of the methyl protons of dethiaAcCoA support the same conclusion (*13*).

Consideration of the citrate exchange data (Table 4, Figure 5) taken together with the steady-state kinetic data (Table 1) for H320Q, G, and N reveals an apparent paradox. Rates and extents of exchange vary inversely with the values of k_{cat}. Very little exchange is observed for H320Q and nearly complete exchange for H320N. The K_m values, which are all substantially greater than those found for WT, also vary directly with the rates and extents of exchange; the enzyme with the lowest affinity for substrates (H320Q) also shows the least exchange. The H320 mutants obey a rapid equilibrium mechanism with respect to substrate binding, and the K_m values represent true dissociation constants. There

[5] Indeed, the expected burst phase is observable under stopped-flow conditions for H320Q, G, and N. Data can be collected only at 236 nm where thioester disappearance can be detected on top of considerable absorbance from the adenine and protein chromophores. The second-order DTNB reaction which monitors CoA product appearance at 412 nm is too slow to monitor the rate of fast transients. For WT and D375E, it is possible to show identity between the (relatively slow) transient in data collected at 236 and 412 nm and thus show that substrate disappearance has the same time course as product appearance; an experiment not possible for H320Q, G, and N. Nonetheless, the H320Q, G, and N transients share many properties with those observed for the WT transient. They are readily fit either by a single exponential decay (at high enzyme concentrations) or by a single exponential followed by a linear decay (at lower enzyme concentrations). The transient amplitudes are directly proportional to [E] but independent of [S] (as long as [S] is in excess). Using the extinction coefficient for substrate disappearance, the amplitudes exceed the enzyme concentrations by severalfold in all cases and thus represent several turnovers. The transient rate constants are directly proportional to [E] and [S] with no signs of saturation. However, for H320R, the rapid transient at 236 nm shows an increased absorbance and thus could not represent substrate utilization. It was not possible in the case of H320R to increase the enzyme concentration to a level sufficiently high so that the transient comprised the entire time course. While the amplitude was directly proportional to [E], the rate was roughly independent of it. We conclude that the 236 nm transients for H320Q, N, and G do in fact represent the same rapid product formation phase as in WT but occurring on faster time scales, while the transient observed with H320R primarily results directly from a conformation change. Even for H320Q, N, and G, we cannot exclude a contribution at 236 nm from other processes such as a conformation change, formation of an enzyme covalent intermediate with a slightly different absorbance spectrum, etc.

is no doubt that the rate of dissociation of AcCoA for these mutants is fast, of the order of 10 000 s^{-1}. The simplest explanation for the wide range of exchange extents is that the value of the rate constant for the step following proton transfer, condensation (k_4), varies from about 10 times faster to 10 times slower than that for the reverse of the proton transfer (k_{-3}). [This conclusion is obtained using a set of reasonable rate constants compatible with the kinetic constants to simulate (Kinsim (*35*)) the observed extents and rates of exchange for the H320 mutants.] Changes in the relative commitments of steps within the condensation sequence account for the observed extents of exchange while the actual rates remain low.

Relative Effects on the Stabilities of Intermediates and Transition States. Condensation is Rate Determining. CitCoA hydrolysis with these mutants is faster than the overall k_{cat} showing that the slowest step is in the condensation reaction. The lack of partitioning of the chemical intermediate (eq 2) back to reactants ($\leq$3%, Table 3) shows that reversal of the condensation is made practically impossible by any substitutions at this position. The barrier to the actual condensation (k_4) must be raised by the inability of any of these enzymes to polarize the carbonyl of OAA. *The k_{cat} for H320X is the apparent forward rate constant for the condensation step, f* (eq 1). Since k_{-2} is large compared to the other terms in f in the case of a prior-equilibrium mechanism, eq 2 reduces to the ratio r/k_5. Although cooperativity prevents assignment of an exact value for k_5, its value is clearly smaller for the mutants than for the WT. Thus, the numerator of eq 2, *r*, which represents the reverse step of the condensation reaction, must be *very* small for the H320 mutants. This term can be dropped from the k_{cat} for these mutants. Since k_5 still remains substantially faster than condensation for all the H320X, we can write eq 3.

$$k_{cat} = f = \frac{k_3 k_4}{k_4 + k_3 + k_{-3}} \qquad (3)$$

Quite reasonably, the residual relative effectiveness of the mutants in carbonyl polarization is reflected in the order of the k_{cat}s as well as in the order of the *relative* values of the condensation and reverse proton-transfer rate constants, k_4/k_{-3}. Modeling studies (S. J. Remington, personal communication) reveal that a glutamine side chain can form all the hydrogen bonds of the naturally occurring imidazole without generating any bad contacts. The otherwise open space left by the glycine side chain could readily be filled by a water molecule also satisfying the hydrogen bonds. The asparagine side chain is least satisfactory; it is too short to hydrogen bond to the carbonyl and too large to admit a water molecule. The structural predictions track well with the kinetic and exchange data.[6]

Hydrolysis. The CitCoA hydrolysis rates for H320Q, G, and N are also less than observed in WT in either its burst or its steady state showing that even the hydrolysis reaction is deleteriously affected by a mutation that primarily slows the condensation. This is also true for H320R, which shows a normal progress curve shape and normal concentration dependencies of initial velocities (no substrate inhibition). Even for H320R which catalyzes hydrolysis at a fast rate compared to its ability to catalyze condensation, its k_{cat} for CitCoA hydrolysis is even slower than the *inhibited* rates of any of the other enzymes (Figure 3B).

The deleterious effects of the H320 mutations on the forward CitCoA hydrolysis rates could originate in disturbances of hydrogen bonding to the CitCoA intermediate, since the $-$OH of CitCoA hydrogen bonds to H320. Perhaps the binding energy provided by these interactions has been utilized by the enzyme to stabilize the transition state(s) for the hydrolysis reaction. Note that the rates of CitCoA hydrolysis follow the same order as the ability of the mutant side chains to hydrogen bond (Figure 3). Since several of these mutants (G and N) cannot have a (direct) hydrogen bond to the citryl-CoA alcohol, it is unlikely that this effect is due to excessive stabilization of that intermediate, but is primarily a result of transition state destabilization.

The OAA site mutants at H320 have functional defects in both their ability to activate OAA through carbonyl polarization and to activate AcCoA by efficient catalysis of proton transfer from its methyl group, but these failures do not seem to be a consequence (except possibly in the case of H320R) of a gross failure to form the "closed" form of the enzyme.

AcCoA Site: D375E and G

Conclusions from Kinetic and Inhibitor Constants. The significantly increased affinity of D375 mutants for the substrates in comparison with WT suggests that some of the ground-state substrate interactions with this residue are energetically unfavorable and may represent substrate destabilization normally relieved in the transition state. Confirming this conclusion from kinetic constants, D375E binds the ground-state analogue more tightly than the WT but the enol(ate) analogue 100-fold more loosely. D375G binds both the ground-state and enol(ate) analogues very much more

[6] In principle, the rate of exchange of the hydrogen transferred to the active-site base with the deuterons of the solvent could be kinetically significant, even rate determining. There is general agreement that all the chemical steps of the catalytic cycle occur in the closed form. In the closed form crystal structures, the active-site base does not have access to the bulk solution through a water "channel" (*3*, *5*, *6*). However, the second carboxyl oxygen of D375 is hydrogen bonded to a water molecule which in turn is hydrogen bonded to a complex polar network through which we can imagine indirect access to the bulk solution. What seems to be at issue is whether the protonated Asp would have time and freedom to rotate so that such an exchange process could operate efficiently. Thus, it may be unnecessary for the enzyme to "open" in order for exchange to be rapid. Even if "opening" is required, it may occur on a time scale fast compared to reaction for the slow mutant enzymes. For TIM, "opening" occurs at a rate of 3×10^4 s^{-1} (*7*). The extreme position, which holds that the range of solvent exchange observed in the H320 mutants is completely controlled by the relative abilities of the mutants to stabilize the closed form, is unlikely to be correct. The data suggest that there is no defect in the ability of these mutants to "close". The CD changes arising from the adenine chromophore (Figure 2) which accompany ternary complex formation for H320Q, G, and N are the same as those for WT. In contrast, the H320R ternary complex spectrum is identical to that of the binary complex. Indeed this enzyme may not be able to "close" as a consequence of the steric bulk or the positive charge of the side chain. More convincing evidence comes from the lack of NMR chemical exchange (Table 2) between free and bound CMCoA ternary complexes of the H320Q, G, and N mutants. Experiments to probe more definitively the dynamics (open-closing rate) as well as the position of the equilibrium between the forms as affected by the mutations are needed. Major projects using solid-state NMR (analogous to ref *7*) as well as fluorescence-transfer experiments using labeled enzymes may give more quantitative information on the rate and position of equilibrium of the closed-open CS conformation change.

tightly than WT (data were insufficiently precise for this very tight binding mutant to be able to quantify these observations). For D375G, some of this increased affinity may be electrostatic in origin. See extensive discussions in refs *1* and *36*.

Effects on the CS Conformation Change. The relatively small changes in the CD spectra of binary and ternary complexes of these mutants suggest little or no perturbation of the ability of to achieve the closed form. This conclusion is further strengthened by slow chemical exchange of CMCoA out of ternary complexes and the presence of marked kinetic cooperativity when CitCoA is utilized as substrate by D375E.

Effects on OAA Activation (Carbonyl Polarization). The mutants in the AcCoA-binding site behave very differently from those in the OAA site. As indicated by the deshielding of the OAA carbonyl chemical shift, *both D375G and D375E achieve full polarization of the OAA carbonyl in ternary complexes*. Thus, all the H320 mutants have a major impact on the AcCoA site interactions, but the converse is not the case. Therefore, polarization, not just binding of OAA, is necessary for generating the function of the AcCoA site.

Effects on Ability to Catalyze Proton Transfer from AcCoA. In comparison to the H320 mutants, the larger chemical shift changes of the carboxyl of the enolate inhibitor suggest that more profound disturbances of hydrogen-bonding environment occurs in D375 mutants. The full mechanistic significance of these larger changes are presently unknown.

D375 is the base removing the proton from AcCoA. This is why the rate of exchange of solvent protons catalyzed by D375E into AcCoA is low (Table 4, somewhat slower than the reaction itself). The importance of D375 is further illustrated by the D375G mutant which tightly binds substrates and analogues but is unable to catalyze *any* of the partial citrate synthase reactions.

Relative Effects on the Stabilities of Intermediates and Transition States. Hydrolysis is Rate Determining. D375 mutants dramatically disrupt the internal thermodynamics of the enzyme. For the WT, the partitioning of the intermediate is consistent with a reaction profile having relatively equal barriers to forward and reverse condensation and hydrolysis (*23*). For D375E, the initial partitioning of CitCoA favors the reactants, indicating a *relatively* larger increase in the barrier height for the hydrolysis step than for the condensation step [similar results have been found for the *E. coli* enzyme which has most of the active site residues strictly conserved (*37*)]. Hydrolysis has become the slowest step by far.

This is the reason that the final acceleration phase for D375E when CitCoA is the substrate is so large that it dominates the progress curves. The high affinity of the enzyme for CitCoA combined with its inhibited steady-state rate of turnover is the cause of the terminal acceleration phases for both WT and D375E. As long as significant CitCoA is left in the solution, its extraordinarily high affinity for the enzymes compared to the normal substrates prevents much processing of the OAA/AcCoA which builds up during the reaction from the reversal of the condensation reaction (*15*, *23*). Thus, the final acceleration phase in both these enzymes results from the feed through of the OAA/AcCoA produced during the steady-state of CitCoA hydrolysis. [Because no OAA is produced from CitCoA in the reactions catalyzed by H320X (see above), no final acceleration phase occurs.] In the WT, the effective barrier for cleavage is higher than the barrier for hydrolysis and most of the CitCoA goes directly to citrate/CoA so the amplitude of the final phase is small. A dramatic reversal of this situation occurs in D375E where the barrier to hydrolysis is much greater than the barrier to cleavage.

The dominance of the internal reaction profile by the barrier to the CitCoA hydrolysis reaction also explains the citrate exchange results (Figure 5). When the reaction is started with AcCoA, we see nearly complete exchange into citrate since the reaction backs up behind the very high barrier to hydrolysis and many cycles of reversal through the condensation pathway result. If the reaction is started from CitCoA, a pattern emerges which allows an interesting distinction. If OAA release is allowed to be reversible, a bimodal distribution of isotopes results with relatively high peaks at d_0 and d_2. The citrate with d_3 and d_4 must have cycled back to free OAA whose methylene was then free to exchange nonenzymatically. At least some of the d_2- and d_3-citrate results from exchange from only the bound form of reactants, and the d_0-citrate comes from the approximately 10% of the CitCoA which is directly hydrolyzed to citrate without any opportunity to exchange by reversal. If any return of free OAA from solution is prevented by trapping it with cMDH/NADH, all the exchange occurs while ligands are bound to the enzyme and only d_0-, d_1- and d_2-citrates are found. The deuterium excess under these latter conditions is about half that when CitCoA is allowed to cycle all the way back to free OAA (Table 4). Thus, about half the exchange occurs when the reactants are enzyme bound. [A similar but less dramatic effect is seen in the WT where the higher degrees of exchange (d_2 in this case) are only seen if OAA is allowed to cycle through the free state (Table 4, Figure 5)].

For D375E, k_5 (eq 1) Is Very Small. Since the initial partitioning of the chemical intermediate indicates a *very high barrier for the hydrolysis step.* There is convincing evidence from the exchange experiments with dethiaAcCoA (*13*) that the proton-transfer step (k_3) is substantially defective in this mutant. This suggests that the term f is small compared to the AcCoA dissociation rate constant (k_{-2}), in which case the partitioning (eq 2) is dominated by the ratio of the apparent rate constant for CitCoA cleavage to that for hydrolysis: $r/k_5 = 8$. If k_{-3} is large compared to k_3 and k_4, i.e., a very unstable proton-transfer intermediate, this term further simplifies to $k_{-4}/k_5 = 8$.

These D375 mutants of a single residue show two functional failures. They cannot efficiently abstract the proton from AcCoA nor can they efficiently hydrolyze the CitCoA intermediate. However, mutating D375 does not affect OAA binding or polarization.

OAA/AcCoA Site: H274G

Conclusions from Kinetic and Inhibitor Constants. Mutations in H274 solely affect transition state-stabilization; the K_ms for this mutant are close to those observed for the WT while the k_{cat} is greatly decreased. As expected, then, only the binding of the enol(ate) analogue is deleteriously affected and not that of the ground-state analogue (dethiaAcCoA), implying that the hydrogen bond provided by H274 to the

AcCoA carbonyl is more important to the stabilization of the enol(ate) intermediate than it is to the ground-state thioester.

Effects on the CS Conformation Change. The response of the CD spectrum of H274G to OAA binding is indistinguishable from WT. (H274G is the only mutant found to behave totally normally in this regard.) The ternary complex spectrum is similar to that for WT and D375E. Since this mutant is unable to polarize OAA (from NMR results), CD spectral changes which normally occur on OAA binding are not directly linked with carbonyl polarization. Again, the closed form may be more difficult to reach, but its final structure seems to be very little perturbed at least as detected by this method. As in the other mutants, the ternary complex is in slow exchange on the NMR time scale, indicating *relatively* slow dissociation of the ligand from the complex.

This mutant shows no kinetic cooperativity with CitCoA as substrate. In our model, this means that the closed form is not accessible or that the interconversion between open and closed forms is fast even in the presence of a high-affinity CitCoA. The first possibility is ruled out by the CD and NMR data discussed in the preceding paragraph. H274 hydrogen bonds to the carbonyl of CitCoA. From the relative ease of the cleavage reaction of CitCoA with this mutant, it is apparent that the bound CitCoA is relatively destabilized in comparison with its stabilization in the other enzymes. This destabilization may be sufficient to avoid the substrate inhibition observed with the other mutants whatever the detailed allosteric mechanism. For H274G, the overall situation is complex since H274 is both a catalytic and hinge residue (showing a large change in coordinates between the open and closed form of the enzyme). [It will be of interest in the future to examine the pure hinge mutants (G275A and G275V, (*11*)).] What effect substitution of a glycine residue has on the conformational equilibrium and what part of the catalytic defect may be attributed to perturbation of that equilibrium are subjects of future work.

Effects on OAA Activation (Carbonyl Polarization). From the NMR data (Table 2) *H274G shows two binding modes of OAA in the binary complex and polarization is not achieved in either binary or ternary complexes (1).* In the binary complex, the two binding modes interconvert only very slowly (k_{exch} must be $\ll 76\ s^{-1}$, the separation between the OAA resonances). Only one resonance is observed in the ternary complex, indicating one binding mode. However, for H274G, polarization is still not achieved. The structural data (*38*) suggest that replacement of H274 with a small glycine residue is likely to open up a space allowing a greater freedom of motion for the OAA molecule than it would otherwise experience.

Effects on Ability to Catalyze Proton Transfer from AcCoA. The rate of exchange of solvent protons into AcCoA or dethiaAcCoA (*13*) is very low indicating that failure to stabilize the enol(ate) intermediate by the hydrogen bonding normally provided by H274 has inevitably slowed its formation.

Relative Effects on the Stabilities of Intermediates and Transition States. Condensation Is Overwhelmingly Rate Determining. We can calculate f and r for H274G (eqs 1 and 2). Substantial partitioning of the intermediate is observed. Knowing the value of k_5, 150 min^{-1}, allows us to calculate that the numerator of eq 2, and the effective rate constant for the reverse of the condensation, *r*, has a value of 30 min^{-1}. Together with the value of k_{cat} (Table 1), we calculate a value of 1.2 min^{-1} for the effective rate constant for the forward condensation, *f*. The proton-transfer step (k_3) is profoundly damaged (*13*). The removal of a hydrogen bond to AcCoA and its activated form greatly impedes the necessary proton transfer (*13*). The NMR experiments show that this mutant cannot polarize the carbonyl of OAA so the condensation step (k_4) is impaired as well. This opening up a space in the back wall of the OAA-binding site by substituting G for H interferes with OAA carbonyl polarization; perhaps by compromising the positioning of the OAA carbonyl in a region of appropriate electrostatic potential. [Recall that H274 is both a hinge and catalytic residue and the conformation change is undoubtedly altered. In the future it may be possible to disentangle these effects by studying a pure hinge mutant such as G275V (*11*).]

Since this mutant has a very much higher barrier to the forward condensation than it does to the hydrolysis of CitCoA and since the cleavage is relatively facile, the OAA and AcCoA produced from CitCoA are essentially trapped and cannot be utilized until all the CitCoA is processed. This gives rise to marked two-phase kinetics for CitCoA utilization by this mutant. It appears that the forward and reverse condensation and hydrolysis barriers are also increased over those of WT, but the overwhelming defect in this mutant appears to lie in the early, proton-transfer step.

Hydrolysis. Hydrolysis of CitCoA is at least 10 times faster than the overall k_{cat} though slower than the k_{cat} of WT. So, although condensation is probably more profoundly affected in this mutant than in the others, hydrolysis does not escape unscathed. The structural basis of the deleterious effects of the H274 mutation on the CitCoA hydrolysis rate probably originates in disturbances of hydrogen bonding to the CitCoA intermediate if the binding energy provided by this hydrogen bond has been utilized by the enzyme to stabilize the transition state(s) for the hydrolysis reaction.

As in the case of the mutants in H320, H274G is affected in all the functions identified for CS catalytic residues.

Conclusion. Is the Citrate Synthase Condensation Reaction Ever Concerted? When Is There an Intermediate? The results reported here have allowed a partial dissection of the roles of three catalytic residues of citrate synthase. The energetics of the catalysis are tightly coupled with each residue having important interactions in each stage of the catalytic cycle. It is remarkable that changes in H320, which apparently only interacts with OAA, have such profound affects on AcCoA activation. Were there no evidence to the contrary, the easiest explanation would be that the condensation reaction is concerted with proton transfer and carbon−carbon bond formation occurring simultaneously. This is clearly not the case for the H320Q, N, and G mutants where solvent exchange occurs before generation of CitCoA whose formation is not reversible. However, if the energy well for the enol(ate) intermediate is a relatively shallow one, then the flanking transition states for proton transfer and carbon−carbon bond formation are close in energy and structure [Hammond Postulate (*39*)]. Any structural perturbations destabilizing one transition state will also affect the other. When we consider that the enzyme catalyst is an inseparable component of both chemical transition states, then it is even more reasonable that perturbations of one part

of the active site will affect all the processes that take place within it. This idea was elegantly elaborated by Jencks in his discussion of the factors controlling the existence of discrete intermediates in several types of organic reactions (*40*).

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