Solid State NMR Studies of Hydrogen Bonding in a Citrate Synthase Inhibitor Complex†

Zhengtian Gu,‡ Dale G. Drueckhammer,§ Linda Kurz,|| Kai Liu,‡ David P. Martin,§ and Ann McDermott*,‡

Department of Chemistry, Columbia University, New York, New York 10027, Department of Chemistry, State University of New York at Stony Brook, Stony Brook, New York 11794, and Department of Biological Chemistry, Washington University, St. Louis, Missouri 63110

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ABSTRACT: The ionization state and hydrogen bonding environment of the transition state analogue (TSA) inhibitor, carboxymethyldethia coenzyme A (CMX), bound to citrate synthase have been investigated using solid state NMR. This enzyme−inhibitor complex has been studied in connection with the postulated contribution of short hydrogen bonds to binding energies and enzyme catalysis: the X-ray crystal structure of this complex revealed an unusually short hydrogen bond between the carboxylate group of the inhibitor and an aspartic acid side chain [Usher et al. (1994) *Biochemistry 33*, 7753−7759]. To further investigate the nature of this short hydrogen bond, low spinning speed ^{13}C NMR spectra of the CMX−citrate synthase complex were obtained under a variety of sample conditions. Tensor values describing the chemical shift anisotropy of the carboxyl groups of the inhibitor were obtained by simulating MAS spectra (233 ± 4, 206 ± 5, and 105 ± 2 ppm vs TMS). Comparison of these values with our previously reported database and ab initio calculations of carbon shift tensor values clearly indicates that the carboxyl is deprotonated. New data from model compounds suggest that hydrogen bonds in a syn arrangement with respect to the carboxylate group have a pronounced effect upon the shift tensors for the carboxylate, while anti hydrogen bonds, regardless of their length, apparently do not perturb the shift tensors of the carboxyl group. Thus the tensor values for the enzyme−inhibitor complex could be consistent with either a very long syn hydrogen bond or an anti hydrogen bond; the latter would agree very well with previous crystallographic results. Two-dimensional $^{1}H-^{13}C$ heteronuclear correlation spectra of the enzyme−inhibitor complex were obtained. Strong cross-peaks were observed from the carboxyl carbon to proton(s) with chemical shift(s) of 22 ± 5 ppm. Both the proton chemical shift and the intensity of the cross-peak indicate a very short hydrogen bond to the carboxyl group of the inhibitor, the C···H distance based upon the cross-peak intensity being 2.0 ± 0.4 Å. This proton resonance is assigned to $H^{\delta 2}$ of Asp 375, on the basis of comparison with crystal structures and the fact that this cross-peak was absent in the heteronuclear correlation spectrum of the inhibitor−D375G mutant enzyme complex. In summary, our NMR studies support the suggestion that a very short hydrogen bond is formed between the TSA and the Asp carboxylate.

Citrate synthase (EC 4.1.3.7) catalyzes the stereospecific condensation of acetyl-CoA[1] and oxaloacetic acid to form citrate in the citric acid cycle (*2*). Scheme 1 indicates the products, reactants, and presumed intermediate of this reaction as well as compounds used in this study that mimic the reaction intermediates. The citrate synthase reaction involves attack of the electron-rich center of an enolate or enol species on an electron-deficient center of oxaloacetic acid. This enzyme has provided a convenient system for studying the thermodynamic importance of hydrogen bonding interactions to enzyme mechanisms and to inhibitor affinities. We report spectroscopic studies of an anomalously short hydrogen bond in a citrate synthase−inhibitor complex.

The first step of the citrate synthase reaction, enolization of the acetyl functionality, is a remarkable reaction in that a very weakly acidic proton on a carbon center is transferred to a weak general base. Based on pK_a values in solution, this proton transfer might be uphill by 14 pK_a units or about 19 kcal/mol. The players for this enolization are very similar to those for the analogous reaction in triosephosphate isomerase. In both enzymes, a carboxylate group from an aspartic or glutamic acid functions as the general base, while an imidazole group stabilizes the developing charge on the enolate's oxygen center. In both cases, the general base abstracts the proton in an anti-periplanar arrangement, and the imidazole appears to be ideally situated for efficient hydrogen bonding. Stabilization of the transition state and intermediate relative to the ground state and products presumably derives principally from interactions with the charge that develops in the transition state as well as

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‡ Columbia University.

§ State University of New York at Stony Brook.

|| Washington University.

[1] Abbreviations: AMX, amidocarboxymethyl coenzyme A; CMCoA, carboxymethyl coenzyme A; CMX, carboxymethyldethia coenzyme A; CoA, coenzyme A; CS, citrate synthase; CS/CMX/OAA, ternary complex of citrate synthase with carboxymethyldethia coenzyme A and oxaloacetate; PEG, poly(ethylene glycol); Tris, tris[hydroxymethyl]-aminomethane.

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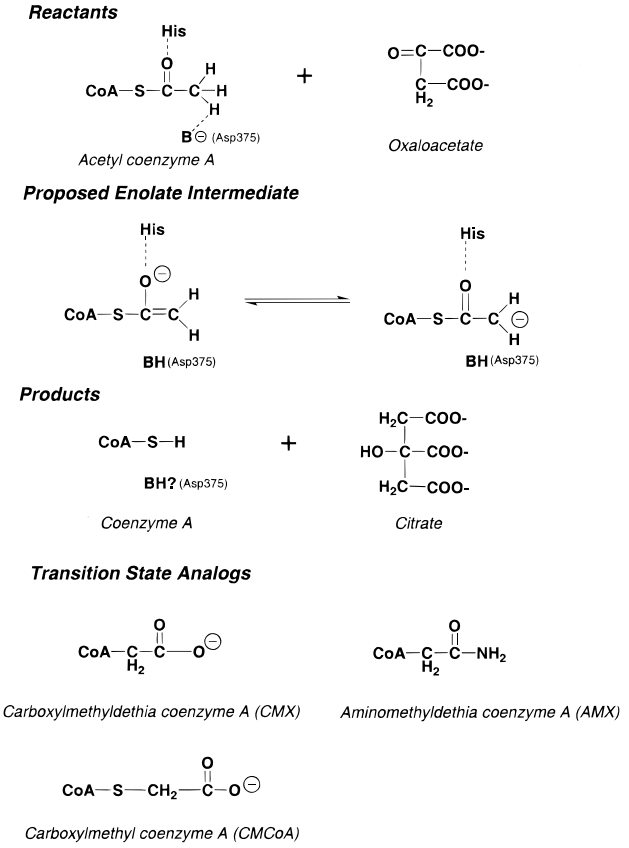
Scheme 1



differences in steric factors and bond lengths. It has been proposed that if the transition state of an enzyme-catalyzed reaction involves hydrogen bonds that are "pK_a matched" while the same hydrogen bonds in the ground state are mismatched, large differences in the hydrogen bond strengths might be anticipated, comparing the ground state and the transition of the reaction. In this context, the term "pK_a matched" refers to two bases competing for a single proton, with equivalent affinities for the proton. It is well known that such arrangements can have anomalously short hydrogen bond lengths in the crystalline state. In the gas phase such species, which typically possess a net charge, can exhibit association energies of more than 30 kcal/mol and have strongly perturbed spectroscopic signatures. Due to the anticipation of extensive proton dynamics, these species have often been described as possessing "low barrier hydrogen bonds". Such "low barrier" or "short strong" hydrogen bonds have been proposed to play a key role in the stabilization of enzyme-bound intermediates in a wide range of enzyme-catalyzed reactions (*21*, *22*). While changes in the strength of hydrogen bonds along the reaction coordinate undoubtedly play a major role in enzyme catalysis, the importance of very short or low barrier hydrogen bonds in enzyme reactions has been widely debated (*23*−*27*). This question has inspired several studies of hydrogen bonding in model systems and in enzyme−inhibitor complexes. Hydrogen bond energies in these systems generally appear modest relative to energies in the gas phase. The situation for the transition state of a reaction differs from that of a long-lived, solvated, and thermally relaxed species in many respects, thus the question of the contribution of low barrier hydrogen bonds in enzymatic reactions is especially difficult to assess. The current study relates to one such case of anomalously short hydrogen bonding in an enzyme−inhibitor complex.

Citrate synthase has been an important case for studying various details of enzyme catalysis including hydrogen bonding. Biochemical, crystallographic, and spectroscopic studies have been performed to elucidate the structure and mechanism of this enzyme, and this work has been reviewed extensively (*3*−*6*). The amino acid sequence (*7*−*9*) and the crystal structures of binary and ternary complexes indicate that His 274, His 320, and Asp 375 are catalytically important amino acid residues (*1*, *10*−*15*). Fourier transform infrared (FTIR), Raman, and nuclear magnetic resonance (NMR) spectroscopy (*16*−*19*) and mutagenesis studies (*20*) have helped to further clarify the mechanism of the condensation reaction. The crystal structures of several citrate synthase−inhibitor complexes have been reported (*1*, *12*, *15*) including the complexes with carboxymethyl coenzyme A (CMCoA), carboxymethyldethia coenzyme A (CMX), amidocarboxymethyldethia coenzyme A (AMX) (shown in Scheme 1), and the α-fluoro analogues of CMX and AMX. These inhibitors mimic the geometry of the enolized form of acetyl CoA, with an oxygen (or nitrogen in AMX) replacing the methyl group and a CH_2 group replacing the thioester sulfur atom (CMCoA has an extra methylene group inserted between the sulfur atom and carbonyl carbon). An unusually short hydrogen bond ($d_{O\cdots O} \approx 2.4$ Å) between the carboxyl oxygen of CMX or CMCoA and the carboxyl oxygen of Asp 375 was observed (*1*). In keeping with the proposed significance of low barrier or short strong hydrogen bonds, these inhibitors exhibit much tighter binding constants than the substrate, with the K_d for inhibitors being in the nanomolar range while the substrate's K_m is in the micromolar range, when measured in ternary complexes with oxaloacetic acid. An increase of only 1−2 kcal/mol in binding energy is observed for CMX, which forms a very short hydrogen bond in the citrate synthase complex, as compared with AMX, which forms a slightly longer and strongly pK_a-mismatched hydrogen bond (*1*, *15*). These binding comparisons were made at pH 6 so that Asp 375 would be protonated, as required for binding of CMX. The goal of the studies described here was to further probe the existence of the short hydrogen bond using structural and spectroscopic insights from solid state NMR, with particular emphasis on the confirmation of presumed ionization states.

The ionization state and hydrogen bonding environment of the carboxyl group of acetyl-CoA analogues in the citrate synthase active site were studied using carbon and proton chemical shifts from solid state NMR. The proton shift is a probe of hydrogen bonding that is almost as old as NMR spectroscopy itself, with deshielded values being associated with very strong or short hydrogen bonds (*29*, *30*). The carbon shift tensor measurements can be directly related to ionization state and hydrogen bonding length for the case of carboxyl groups and provide information that can be complimentary to that from the proton shift (*31*). Carbon δ_{11} values are below 250 ppm for deprotonated carboxyl groups and above 250 ppm for protonated groups (*31*). The δ_{22} element varies dramatically depending on the hydrogen bonding geometry and length (*31*). For the protonated case, a less shielded or larger δ_{22} value corresponds to shorter syn hydrogen bonding of a neighboring proton to the carbonyl while a more shielded or smaller δ_{22} value corresponds to

shorter syn hydrogen bonding for the deprotonated case. The δ_{33} element could presumably be used as an indication of mobility of the carboxyl group. Its value is 110 ± 5 ppm (independent of hydrogen bonding environment or ionization state) if the group is rigid but could be more deshielded due to averaging with the other tensor values if the group is mobile.

We demonstrate with this report that heteronuclear correlation spectroscopy can be used to detect protons in large enzymes, to estimate C−H distances, and to characterize the hydrogen bond environment. Both shift analysis and correlation spectroscopy were used in this study to show that there is a short hydrogen bond in the citrate synthase−CMX complex.

EXPERIMENTAL PROCEDURES

Porcine heart citrate synthase was purchased from Sigma Chemical Co. and used without further purification. The mutant enzyme, D375G, was prepared as previously described (*33*). The purities of both native and mutant enzymes were examined by 10% SDS−polyacrylamide gel electrophoresis (*32*). Natural abundance carboxymethyldethia coenzyme A (CMX) was synthesized as described previously (*34*, *35*). The ^{13}C-labeled CMX was prepared by the same method from 1-^{13}C-4-aminobutyric acid. The anti hydrogen bonding model compounds, imidazole-4-acetic acid hydrochloride and sodium imidazole-4-acetate, were purchased from Sigma Chemical Co. and used without further purification.

The protein samples for solid state NMR experiments were prepared by precipitation with poly(ethylene glycol) (PEG 8,000 from Sigma Co.), as described previously in the preparation of triosephosphate isomerase (*36*). Enzyme activity in the presence of various concentrations of PEG was determined by adding 0.22 μmol of acetyl-CoA to a controlled amount of enzyme (0.2−0.4 units) in Tris buffer (100 mM, pH 8.0) containing oxaloacetate (0.6 μM) and a given concentration of PEG (total volume of 1.5 mL). Activities were then measured by monitoring the disappearance of the thioester absorbance at 233 nm using $\epsilon_{233} = 5.4 \times 10^3$ M^{-1} cm^{-1}.(*37*). The activity of the PEG-precipitated enzyme under conditions similar to those studied by NMR was confirmed as follows. Precipitation by incubation at high concentrations for at least 30 min, followed by rapid dilution into PEG buffer, allowed for preparation of dilute precipitated protein, specifically a concentration range appropriate for assays (1−5 μg/mL). The fact that the protein is active under these conditions could be confirmed by a straightforward activity assay in the presence of the PEG as described above; the fact that it remains as a microcrystal or a precipitate for 10 min could be verified by low speed centrifugation (10000*g*, 10 min); upon centrifugation the activity is associated predominantly with the pellet rather than the supernatant.

Two protocols were used to prepare solid state enzyme−inhibitor complexes. In the *incubation* method, 1 equiv of the inhibitor was added to a suspension of precipitated citrate synthase in Tris buffer (0.5 M, pH 8.0) containing PEG (25%), and the mixture was incubated at 4 °C for 2 h. In the *coprecipitation* method, citrate synthase was dissolved in Tris buffer (0.1 M, pH 8.0), and oxaloacetate (2 mM) and inhibitor (1 equiv) were added followed by addition of PEG to a final concentration of 25%. For enzyme samples at pH 6.0, citrate buffer (0.5 M) was used.

Solid state NMR spectra were acquired using a Chemagnetics CMX400 spectrometer, operating at 99.70 MHz for ^{13}C and 396.5 MHz for ^{1}H, and a Chemagnetics 5 mm APEX probe. All spectra of biological samples were recorded at −15 °C to −25 °C. Carbon spectra were acquired using a standard cross-polarization pulse sequence with a contact time of 3 ms, 3.9 μs 90° pulse lengths for protons and carbons, an acquisition time of 20 ms, a recycle delay of 3 (for proteins and a recycle delay of 120−300 for model compounds), and magic angle spinning with speeds as indicated. Approximately 6000−12 000 transitions were accumulated for each protein sample, and 320−600 scans were acquired for each model compound. Adamantane was used as a chemical shift standard, with the more deshielded peak set to 38.6 ppm. The principal values of the chemical shift tensors were extracted by computer simulation of difference spectra, i.e., ^{13}C-CMX-containing ternary complex minus ^{12}C-CMX-containing ternary complex (*39*, *40*). Several different spinning speeds were used to analyze experimental errors and to assign center peaks.

As in previous work (*41*), BR-24 (*42*, *43*) was used to suppress proton homonuclear couplings during t_1, the ^{13}C−^{1}H heteronuclear dipolar coupling was reduced during the t_1 period by ^{13}C-BB-24 decoupling (*44*), while WIM-24 (*45*) was used to transfer polarization selectively from protons to carbon spins via the heteronuclear dipolar interaction, with both ^{1}H and ^{13}C homonuclear dipolar couplings suppressed. Cross-polarization times were typically 125 μs or 170 μs, as indicated. Two-dimensional (2D) spectra were collected with 40 points in the t_1 period or ^{1}H dimension (zero-filled to 512 points), and 512 points were in the t_2 period or ^{13}C dimension (zero-filled to 1024 points). TPPI phase cycling was used to obtain pure phase 2D spectra, and the proton carrier frequency was usually shifted about 1−2 kHz during t_1. The proton chemical shift was calibrated, including the scaling factor, by measuring a known chemical shift standard (monoethyl fumarate) at several carrier frequencies. The ^{1}H and ^{13}C 90° pulse lengths were 3.5 μs (calibrated within <0.1 μs using multiple-pulse tuning sequences (*46*)). The MAS speed was 2.38 kHz so that $\tau_r > \tau_{mp}$ where τ_{mp} is the multiple pulse time (24 × 3.5 = 84 μs) and τ_r is the rotor cycle time (420 μs). The delay between transients was 2.5 s, and 1024−2048 scans were accumulated for each t_1 point. About 10−20 mg (0.2−0.4 μmol) of protein sample was used for each spectrum, and the 2D spectra were acquired in 1−2 days.

Proton spectra of the solid state samples were acquired using the windowless BR-24 (*42*, *43*) pulse sequence. Pulse lengths of 3.5 μs (90° pulse) with "τ" values of 3.5−4.0 μs and 3−120 s recycle delays were used; 16−32 scans were accumulated. Signal averaging of 16−32 scans per increment was utilized.

RESULTS

Purities of both wild-type citrate synthase and the D375G mutant were determined by electrophoresis and shown to be greater than 95%. The major impurity bands were degradation products that do not bind the inhibitor, as confirmed by active site titration (*33*). Enzyme samples for solid state NMR experiments were prepared by precipitation with poly(ethylene glycol). Citrate synthase activity was determined at different PEG concentrations as shown in

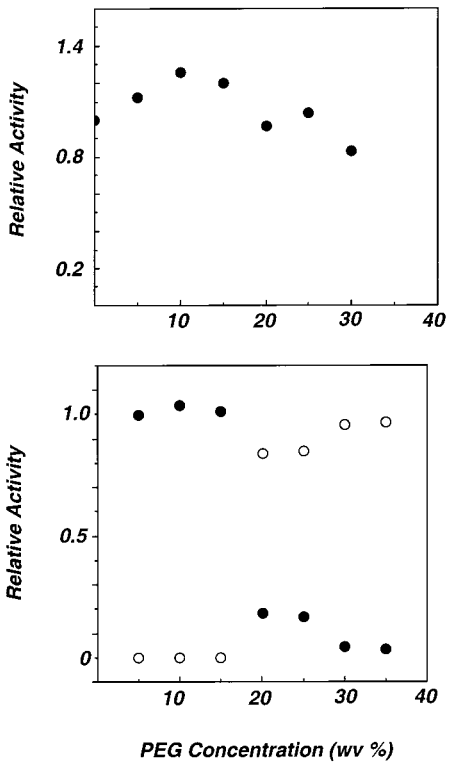


FIGURE 1: The enzymatic activity of citrate synthase is plotted as a function of PEG concentration. Part a shows the activity of the enzyme in the presence of various PEG concentrations, while part b shows the activities of supernatants and precipitates. Open and closed circles represent data for precipitate and supernatant, respectively.

Figure 1. Specific activity was 195 units/mg protein ± 20% over a PEG concentration range of 0−30%. Activity assays were also performed before and after centrifugation of aliquots of the enzyme suspension in the presence of PEG. After centrifugation the enzyme was suspended in PEG-containing buffer, whereupon it remains a solid for many minutes (slow dissolution kinetics) and therefore the solid form of the enzyme can be monitored for activity. These data in aggregate illustrate that citrate synthase is precipitated in the presence of 20% PEG and forms a solid, that PEG does not interfere with enzymatic activity, and furthermore that the solid form of the enzyme is as active as the solution form. As described below, state enzyme−inhibitor complexes were prepared both by diffusion of inhibitor into precipitated enzyme and by coprecipitation of enzyme in the presence of inhibitor.

The ^{13}C-labeled carboxyl group in the protein−inhibitor complex gave rise to a spectrum with one sharp center band peak at 181.9 ppm for samples prepared by coprecipitation. Figure 2a depicts the carbon spectrum of ^{13}C-labeled CMX bound to citrate synthase in a ternary complex with oxaloacetate. Figure 2b depicts the spectrum of an analogous complex involving unlabeled (natural abundance) inhibitor, while Figure 2c depicts the difference spectrum (a − b). Chemical shift tensor values can be simulated from the intensities of spinning sidebands in c (*39*, *40*) and were determined to be 233 ± 4, 206 ± 5, and 105 ± 2 ppm vs TMS (summarized in Table 1). Spectra were obtained at pH 8.0 and 7.0 in Tris buffer and at pH 6.0 in citrate buffer (*32*). Citrate proved to be a poor buffer for precipitating the enzyme−inhibitor complex: higher PEG concentrations (more than 30%) were required to achieve precipitation, and the samples appeared to be less homogeneous on the basis of line widths and intensities. Despite these differences, the isotropic chemical shifts and apparent chemical shift anisotropic tensors of the carboxyl groups of CMX were essentially invariant across the sample conditions.

Carbon NMR spectra of samples prepared by soaking (incubating) the precipitated enzyme−oxaloacetate complex with inhibitor at pH 8.0 (data not shown) (*32*) using cross-polarization from protons to carbon and low speed magic angle spinning. The labeled-minus-unlabeled low speed difference spectra exhibited two species. Shift tensor analysis of these two species is reported in Table 1. These data indicated an apparently nonspecifically bound species with an isotropic shift of 183.5 (and tensor values of 249 ± 1, 196 ± 1, and 105 ± 1 ppm). The second species has shift tensor values that match those seen for samples prepared by coprecipitation, with an isotropic shift of 181.8 ppm (and tensor values of 230 ± 5, 210 ± 5 and 105 ± 1 ppm); we assign this line to be associated with the species bound to the active site.

To identify the hydrogen bonding partner for the inhibitor, spectra of the ternary complexes of the D375G mutant enzyme with CMX and oxaloacetic acid were also obtained. In this mutant, the putative hydrogen bonding partner, Asp 375, was replaced by glycine to alter the inhibitor's environment. Spectra on lableled inhibitor complexed with this mutant enzyme are shown in Figure 3. Panel a shows the spectrum of D375G citrate synthase with ^{13}C-labeled inhibitor, and panel b shows the labeled-minus-unlabeled difference spectrum, emphasizing the peaks due to ^{13}C-CMX. The difference spectrum exhibits several peaks associated with the protein, indicating a poor subtraction. The peaks in the carboxyl region associated with the inhibitor are sharp and appear with an isotropic shift of 177.7 ppm. The values of the chemical shift tensor elements, 240 ± 3, 188 ± 3, and 105 ± 3, indicate a deprotonated carboxyl group with shorter or stronger hydrogen bonding as compared to the CMX complex with wild-type enzyme.

To provide an explanation for chemical shift values observed in the NMR spectra of the enzyme inhibitor complex, spectra of compounds with anti hydrogen bonding geometries are needed, as the previous database included only carboxyl groups with syn hydrogen bonding motifs (*47*) (Scheme 2). ^{13}C and ^{1}H spectra were determined for one such model compound with short anti hydrogen bonding (*49*). The carbon spectrum was obtained using cross-polarization and magic angle spinning, and the proton spectrum (not shown) was obtained using combined sample rotation and multiple-pulse spectroscopy (BR24). Figure 4a shows the spectrum of imidazole-4-acetic acid hydrochloride, and Figure 4b shows that of sodium imidazole-4-acetate. The chemical shift of the NH proton, the most deshielded peak, in sodium imidazole-4-acetate is around 14.5 ppm. On the basis of the proton spectrum measured using magic angle spinning and multiple-pulse sequences, the imidazole proton forms a very strong anti hydrogen bond to the deprotonated carboxyl group (Figure 4b). Upon protonation of the carboxyl group (Figure 4a), the isotropic chemical shift moves from 180 to 175 ppm. This 5 ppm shift toward the more shielded direction is characteristic of deprotonation. Dashed lines indicate the envelope of the carbon chemical shift tensors,

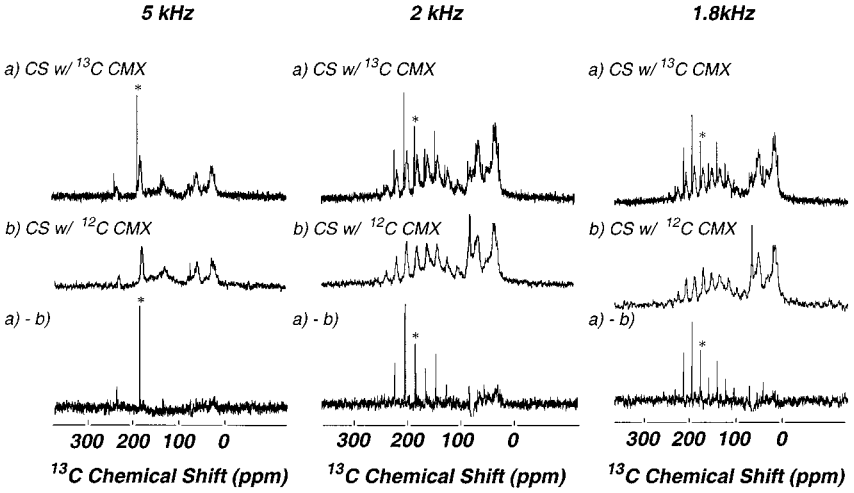


FIGURE 2: Carbon spectra of the ^{13}C-labeled inhibitor in a ternary complex with wild-type porcine citrate synthase and oxaloacetic acid. The spectra were measured using cross-polarization and magic angle spinning and a sample temperature of −20 °C. The top row (a) shows the spectra of ^{13}C-CMX−citrate synthase complexes, while the middle row (b) shows that of ^{12}C-CMX−citrate synthase complexes, and the bottom row (c) shows the difference spectra, a − b. Center peaks are marked with an asterisk. Spinning speeds are as indicated (5, 2, and 1.8 kHz).

Table 1: Chemical Shift Tensors of Carboxyl Groups of Carboxymethyldethia Coenzyme A in the Enzyme−Inhibitor Complexes[a]

	δ_{iso}, ppm	δ_{11}, ppm	δ_{22}, ppm	δ_{33}, ppm
pH 8.0 Tris, unbound (CS complex)	183.5	249 ± 1	196 ± 1	105 ± 1
pH 8.0 Tris, bound (CS complex)	181.8	230 ± 5	210 ± 5	105 ± 1
pH 7.0 Tris, bound (CS complex)	181.6	236 ± 3	202 ± 4	105 ± 2
pH 6.0 citrate, bound (CS complex)	181.9	230 ± 5	220 ± 10	96 ± 5
pH 8.0 Tris, bound (D375G complex)	177.7	240 ± 3	188 ± 3	105 ± 3
protonated with weak syn hydrogen bonding	171	255	150	108
protonated with strong syn hydrogen bonding	178	255	170	108
deprotonated with weak syn hydrogen bonding	182	240	200	105
deprotonated with strong syn hydrogen bonding	174	245	170	107
deprotonated with short anti hydrogen bonding	180	241	195	105
protonated with short anti hydrogen bonding	174	254	157	112

[a] Typical values for carboxyl groups in a variety of environments are based on previous publications[31,47] as well as the data on imidazole acetic acid (Figure 4).

while the values are listed in Table 1. These data support the hypothesis that only syn hydrogen bonding interactions perturb the carbon chemical shift for carboxylate groups.

Two-dimensional ^{1}H−^{13}C heteronuclear correlation spectra of the enzyme CMX complexes are presented in Figure 5. Intense cross-peaks involving the inhibitor's carboxyl group in the carbon dimension and several protons are seen. Cross-peaks associated with the alkyl carbons and protons and the carbonyl carbons and backbone amide protons of the protein backbone are also seen in the labeled complex as well as the unlabeled control. Signals associated with the PEG precipitant are absent in these spectra due to the mobility of PEG at the measurement temperature. The observation of cross-peaks from the carboxyl carbon to proton(s) with a chemical shift around 22 ppm indicates the presence of a very short hydrogen bond to the deprotonated inhibitor (Figure 5). The relative volumes of the inhibitor's cross-peak to the β-methylene protons and to this deshielded proton can be used to estimate the C···H distance. We previously observed that for rigid protons, the heteronuclear correlation cross-peak volumes are related to the inverse cube of the C···H distance (*41*). The cross-peak to the deshielded proton at 22 ± 5 ppm has a peak volume of 0.65 normalized to that of the inhibitor methylene proton peak. Correcting for stoichiometry and assuming a rigid environment, we estimate that the distance from the inhibitor's carbonyl carbon to the deshielded proton is 2.0 ± 0.4 Å. We presume that this short hydrogen bond involves a pK_a-matched partner, since short hydrogen bonds involving deshielded protons generally do involve pK_a-matched species. It is logical to conclude that this partner is the carboxylate side chain of Asp 375.

When aspartic acid 375 is replaced by glycine, the proposed hydrogen bonding between the CMX carboxyl group and aspartate carboxyl is removed. Curiously, the carbon chemical shift measurements indicated that the hydrogen bonding perturbation was stronger in the Gly mutant. To understand this discrepancy, the carbon−proton heteronuclear correlation spectrum of the labeled inhibitor bound to the mutant enzyme was obtained. The deshielded proton seen in the 2D heteronuclear correlation spectrum with wild-type citrate synthase was missing in the heteronuclear correlation spectrum of the labeled inhibitor bound to this mutant enzyme (*32*). On the other hand, the carboxyl carbon of the inhibitor still exhibits strong cross-peaks to the β-methylene protons. Thus the inhibitor's hydrogen bonding partner in the wild-type enzyme is most likely aspartate 375. On the basis of the chemical shift tensor measurements, the carboxylate group has a hydrogen bonding partner. A water molecule might be expected to occupy a cavity created when the Asp 375 side chain is removed and Asp is replaced with Gly; such a water molecule would probably interact with the inhibitor. Alternatively, other side chains might interact

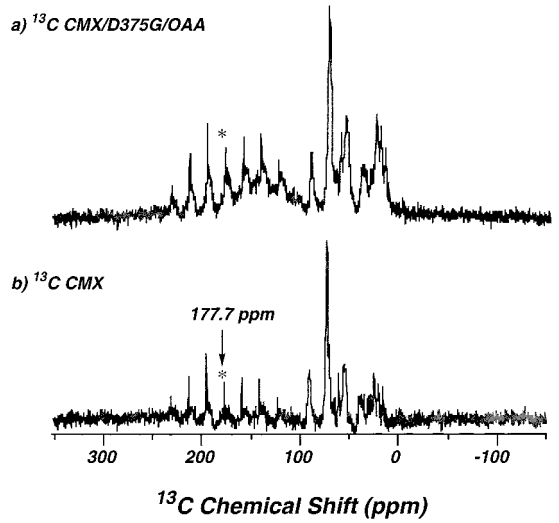


FIGURE 3: Carbon spectra of ^{13}C-labeled inhibitor in a ternary complex with the D-375G mutant citrate synthase and oxaloacetic acid. Spectra were measured using cross-polarization and magic angle spinning with a spinning speed of 1.8 kHz and a sample temperature of −20 °C. The top row (a) shows the spectrum of the ^{13}C-CMX complex, and the bottom row (b) shows the difference spectra of part a and complexes with an unlabeled inhibitor compound. The center peaks are marked by asterisks. Since the background spectra were taken using slightly different conditions, the subtraction shows protein background artifacts.

with the inhibitor, including the catalytic His. In any case, the putative hydrogen bonding partner is not observed in the 2D HETCOR spectrum, possibly because it appears in a congested region or is more mobile.

DISCUSSION

The citrate synthase−CMX complex provides a convenient system for the study of short hydrogen bonds in enzyme active sites. Crystallographic data indicate an exceptionally short hydrogen bond, while binding studies indicate a fairly modest benefit associated with this interaction. The current studies were begun with the aim of verifying the presence of a short hydrogen bond and documenting the ionizations states associated with the enzyme−inhibitor complex. Our NMR data address three points: the ionization state of the carboxylate group of the inhibitor, the length of the shortest hydrogen bond to the inhibitor, and the identity of the hydrogen bonding partner. These three points will be discussed, followed by interpretation of the relevance of these results to the enzyme mechanism and to the understanding of binding constants.

The Ionization State of the Carboxylate Group of CMX in the Ternary Enzyme Complex. Crystallographic results offer poorly defined information on the ionization states of the inhibitor and active site residues. Isotropic carbon chemical shifts in conjunction with pH titrations have been used as a probe to measure the pK_a value and protonation state for many enzymes (*50*−*52*). Detailed solution NMR studies have been performed on the ternary complex of citrate synthase, oxaloacetate and carboxymethyl CoA (CMCoA) (*18*). The chemical shift of 1-^{13}C-CMCoA changes from 178.1 to 175.2 ppm (2.9 ppm more shielded), upon binding to citrate synthase, while free CMCoA exhibits chemical shifts of 178.1 and 174.9 ppm for deprotonated and protonated forms in solution, respectively. The isotropic chemical shift for

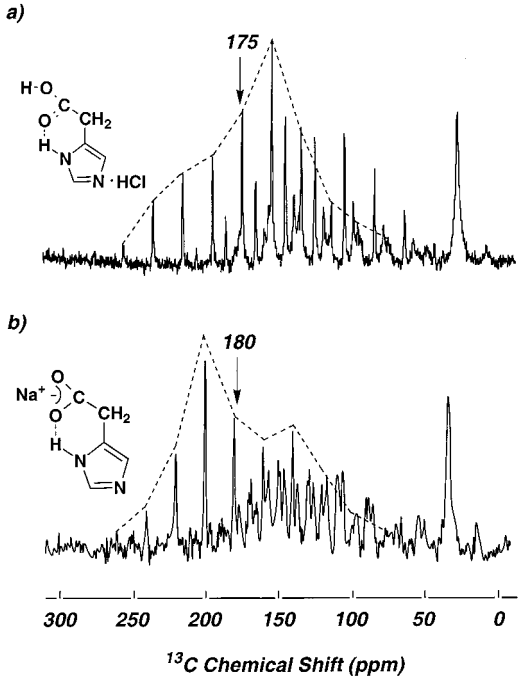
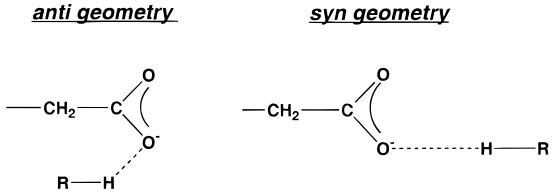


FIGURE 4: ^{13}C spectra of two model compounds with anti hydrogen bonding motifs, measured using cross-polarization from protons and magic angle spinning. Part a shows the carbon spectrum of imidazole-4-acetic acid hydrochloride, which has a protonated carboxyl group, and part b shows that of sodium imidazole-4-acetate, with has a deprotonated carboxyl group. The structures of these compounds are shown next to the spectra, while the dashed lines give the rough tensor shapes. Proton chemical shift and heteronuclear correlation data confirm the strong hydrogen bond (not shown).

Scheme 2



protonated carboxyl groups is typically 175 ppm (*31*) and carboxylate groups normally exhibit 4−6 ppm upfield shifts in carbon chemical shift upon protonation, which is a somewhat larger change than was observed in the CMCoA−citrate synthase complex (*47*). At pH 8.0, the carboxylate group of free CMX is certainly in a deprotonated state, as the pK_a of a typical carboxylic acid is just less than 5 (and α-thio-substituted carboxylate in CMCoA has a pK_a of 3.9 (*18*)). It was concluded that the carboxyl group of CMCoA became protonated upon binding to citrate synthase. Using solid state NMR methods, increased shielding in the isotropic chemical shift was also observed for the carboxyl group of CMX upon binding to citrate synthase, from 183.5 to 181.8 ppm. The shift of the bound form was invariant with pH from 6 to 9. The difference in the absolute shifts observed in the solid state as compared with solution is not clear at present. These shift changes presumably result from the environment provided by the enzyme active site. Since it is not possible to deprotonate this moiety in a titration experiment, the results were not secure, and indeed as we discuss in this work the bound state form is clearly deprotanated and in an unusual hydrogen bonding environment. This

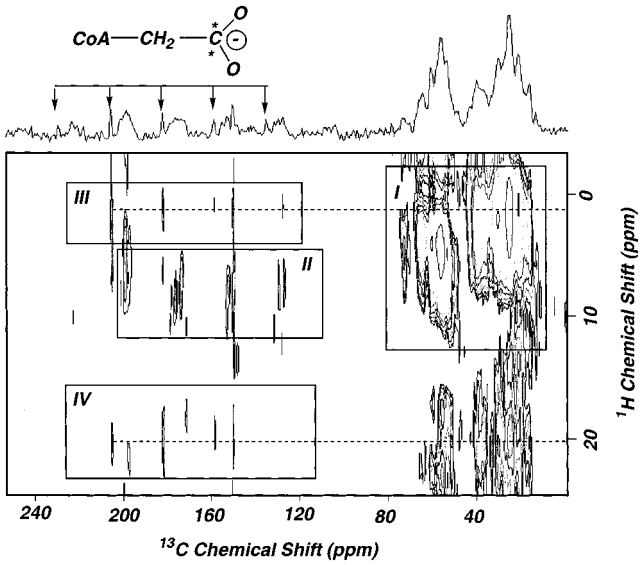


FIGURE 5: Two-dimensional $^{1}H-^{13}C$ heteronuclear correlation spectrum of the ternary complex of porcine citrate synthase with ^{13}C-CMX and oxaloacetic acid. Spectra were acquired at −20 °C. Four cross-peak regions of interest are indicated: (I) protein alkyl carbons to associated protons; (II) protein backbone carbonyl carbons to backbone amide protons; (III) ^{13}C-carboxyl carbon of CMX to methylene protons in CMX; and (IV) ^{13}C-carboxyl carbon of CMX to an acidic proton, presumably from the protein, with a chemical shift of 22 ppm. Spinning sidebands of the ^{13}C-carboxyl group are indicated by arrows. Three WIM24 half-cycles were used, resulting in a contact time of 125 μs. In the 2D $^{1}H-^{13}C$ heteronuclear correlation spectrum of the ternary complex of porcine citrate synthase with ^{12}C-CMX and oxaloacetic acid (not shown), the peaks in regions III and IV are clearly missing.

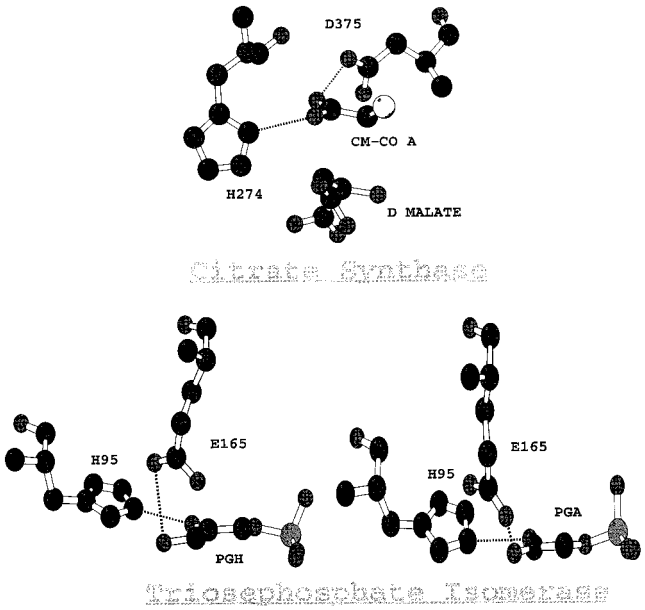


FIGURE 6: Key functional groups in the active site of the ternary complex of porcine citrate synthase with CMX and oxaloacetic acid. Active site structures of triosephosphate isomerase with inhibitor species are shown for comparison (*1*, *12*, *15*). Relative to the enolate mimic compound (carboxylate group), the histidine is in-plane and syn, while the general base aspartic or glutamic acid is anti-periplanar and anti with respect to the Y conjugate group. In these inhibitor complexes the stronger bond involves the inhibitor and aspartate in a thermally relaxed ground state structure; for the proposed transition state, the stronger hydrogen bond is supposed to involve the enolate and the histidine.

example illustrates the confusion that can arise when using database-derived information to study modest perturbations in the isotropic shifts of carboxyl groups.

To analyze the protonation state of the citrate synthase-bound inhibitor, the chemical shift tensor values (Table 1) were examined. The δ_{11} chemical shift tensor value of 233 ± 4 ppm indicates that the carboxyl group of CMX is deprotonated when bound to the enzyme under all conditions studied (*31*). On the basis of previously studied model compounds, the tensor values match those for a deprotonated carboxylate group with very weak and/or long hydrogen bonding.

The line widths of the carboxyl carbon of 1-^{13}C-CMX were less than 50 Hz or 0.5 ppm in the ternary complex with citrate synthase. In our experience, narrow lines can be characteristic of tight or specific binding between the inhibitor and enzyme; thus, this result is consistent with the high affinity of CMX in this timing complex ($K_i \approx 10^{-8}-10^{-9}$ M) (*1*, *15*, *33*). In contrast, weak, nonspecific sites or sites in which significant conformational freedom is present are often associated with inhomogeneously broadened lines. There is no formal or theoretical relation between binding constant and line width, but the line widths for carboxyl groups often reflect sample heterogeneity, and in this case the sharp lines imply a homogeneous environment.

The Hydrogen Bonding Geometry and Environment of the Carboxyl Group in the Enzyme−Inhibitor Complex. The carbon chemical shift results provided no evidence of short hydrogen bonding, in stark contrast with the crystal structure. This dilemma was complicated further by the observation that the carbon exhibits a cross-peak with a proton chemical shift of 22 ± 5 ppm in heteronuclear correlation spectroscopy experiments, a result consistent with a short hydrogen bond. This suggested the need to reexamine the interpretation of the ^{13}C results. Examination of the crystal structure of the ternary complex with the chicken enzyme indicates that the hydrogen bonding geometry of the carboxyl group of CMX has an anti conformation (shown in Figure 6). This hydrogen bonding geometry is not unprecedented, as indicated by a survey of small crystalline hydrogen bonded organic compounds (*53*), but it was not present in the original database of carboxyls used to interpret the ^{13}C chemical shift anisotropy results. For this reason, a search was undertaken for a model compound that does exhibit anti hydrogen bonding with a carboxylate group. Many compounds exhibiting an anti hydrogen bonding geometry have additional conjugated groups, with the intramolecular anti hydrogen bonding typically enforced by the rigidity of these groups (*48*). Additional conjugated groups would perturb the chemical shifts significantly, so imidazole-4-acetic acid was chosen as a model compound which possessed anti hydrogen bonding but no additional conjugated groups. Figure 4 shows the solid state NMR spectra of imidazole-4-acetic acid, and the chemical shift tensor values are listed in Table 1. The shift tensor values in Table 1 are consistent with the carboxyl group of CMX being deprotonated in the citrate synthase complex with a short hydrogen bond in anti geometry to a group in the surrounding environment of the enzyme active site. This unusual geometry might partly explain the unusual changes in isotropic chemical shifts upon binding.

Proton chemical shifts of acidic protons in carboxylic acids have long been established as an indicator of hydrogen bond length and even of their thermodynamic strength (*54*, *55*),

and several authors have suggested that the proton chemical shift is one of the best indicators of low barrier hydrogen bonding (*21*, *56*, *57*). It can be difficult to measure exchangeable acidic protons by solid state NMR, although it has been accomplished in some cases, for example, in a serine protease (*58*). Two-dimensional $^{1}H-^{13}C$ heteronuclear correlation spectroscopy of the solid form of the enzyme gives an opportunity to select the desired proton chemical shift by its correlation to some specific isotopically labeled heteronuclei. The shorter time scale of the solid state measurements (in comparison with solution), together with the lower temperatures used, provide a better opportunity to observe such rapidly exchangeable protons.

A very deshielded proton associated with CMX in the complex indicates the involvement of a very short hydrogen bond. Proton chemical shifts of 22 ppm correspond to an O···O distance of 2.45 ± 0.1 Å (*30*, *55*). It was therefore concluded that the highly deshielded proton lies between the carboxylate oxygen of the inhibitor and the carboxylate oxygen of Asp375, with a distance of 2.45 ± 0.1 Å between the oxygens. The volumes of the cross-peaks in the heteronuclear correlation spectrum also provide approximate distances between two correlated nuclei (*41*). The ratio of the intensity of cross-peaks between the inhibitor ^{13}C carboxyl carbon and acidic protons to that for methylene protons indicated a distance between the carboxyl carbon and acidic proton of 2.0 ± 0.4 Å. These distances, and the proposal of an anti hydrogen bond geometry relative to the inhibitor's carboxyl group, are in agreement with the crystal structure reported by Usher et al. (*1*). The carbon chemical shift, the proton chemical shift, and the intensity of the correlation to the deshielded proton in the 2D $^{1}H-^{13}C$ heteronuclear correlation spectrum give convincing evidence for a very short anti hydrogen bond in the ternary complex.

An interesting comparison is the NMR study on Δ^{5}-3-keto steroid isomerase that also indicated an 18 ppm downfield shifted proton (*48*). Anti hydrogen bonding geometries have also been observed in several other instances, such as in the case of binding complexes of SH3 domain of *c*-Crk with peptides (*59*). This binding site involves three carboxylate groups hydrogen bonding to the amino group of lysine in the peptide. Only one carboxylate group exhibits a syn geometry relative to the amine group and is critical for binding, while the other two carboxyl groups show an anti geometry but have little or no effect on binding.

We thus conclude that the CMX−aspartic acid interaction in citrate synthase is characterized by an unusually short hydrogen bond that is in an anti arrangement with respect to the carboxylate group of the inhibitor. This supports the crystallography results of Usher et al. but does not directly addresses the question of energetics.

Use of a Citrate Synthase Mutant To Address the Role of Asp 375 in Inhibitor Binding. Several active site mutants of citrate synthase have been previously reported. The mutant in which aspartic acid 375 is changed to glycine provides an excellent system to study the hydrogen bonding effects on inhibitor binding. This D375G mutant is essentially inactive, as the catalysis rate is at least 10^{5}-fold slower than that of the wild-type porcine enzyme (*33*). However, the D375G mutant binds CMCoA with a $K_d < 3$ nM in a ternary complex with oxaloacetic acid, which is no more than 2-fold higher than the K_i for CMX (1.6 nM) with the wild-type enzyme at pH 6. The D375G mutant exhibits much stronger binding of CMX relative to the wild-type enzyme at higher pH, as the Asp 375 carboxylate group that must be protonated for CMX complex formation is removed. The isotropic chemical shift of the carboxyl carbon in the ternary complex with the mutant enzyme is 177.7 ppm, 3.6 ppm more shielded than that in the corresponding wild-type complex. This is the lowest chemical shift among all the CMCoA−enzyme complexes studied, and relatively low among carboxyl groups in general (*33*). The fact that the isotropic chemical shift of the 1-^{13}C-CMX carboxyl group bound to the mutant enzyme is 4.1 ppm more shielded than that bound to the wild-type is consistent with solution NMR results in which the mutation also causes a 3.6 ppm upfield shift in bound CmCoA, from 175.2 ppm for the wild-type to 171.6 ppm for the mutant (*18*, *33*).

The chemical shift anisotropy of the carboxyl group in the ternary complex with the mutant enzyme (233 ± 4, 206 ± 5 and 105 ± 2 ppm as shown in Table 1) indicates a deprotonated carboxyl group with weak syn hydrogen bonding. On the basis of the crystal structure of the ternary complex with the chicken enzyme, the carboxyl group of the inhibitor in the ternary complex with the D375G mutant enzyme complex is expected to have one hydrogen bond with His 274 in syn geometry and an anti hydrogen bond between the same inhibitor oxygen and an active site water molecule. However, the anti hydrogen bond to Asp 375 is absent due to the mutation from aspartate to glycine. That the carboxyl group of CMX is deprotonated confirms that Nδ1 of His 274 is protonated in the inhibitor complex, and these two groups appear to be weakly hydrogen bonded. The 2D $^{1}H-^{13}C$ heteronuclear correlation spectrum of the complex with the mutant enzyme further indicates that the carboxyl group of the inhibitor is deprotonated and not engaged in any short hydrogen bonding, since there are no cross-peaks from the carboxyl carbon of the inhibitor to protons with chemical shifts of 10 or greater.

Implications of the Short, Anti Hydrogen Bond and the Deprotonated Carboxylate Group in the Enzyme Mechanism. The finding that the inhibitor is deprotonated when bound to the enzyme over a broad range of sample conditions is consistent with the model that an enolate rather than an enol is an intermediate in the reaction, though this interpretation must be tempered by the greater basicity of the enolate relative to the carboxylate of CMX and thus the greater tendency of the carboxylate to exist in ionized form (*60*). The proposal that an enolate intermediate is more energetically favored is also supported by recent computational studies (*61*).

Computational studies suggest that protonation of a carboxyl group in an anti orientation is less favorable than protonation in a syn orientation by a factor of $10^{3}-10^{4}$ (*62*, *63*). The hydrogen bonding in this system involves an anti−syn combination. The short hydrogen bond and deshielded proton suggests that the two partners are nearly pK_a–matched, despite the asymmetric geometry.

The situation for the transition state or intermediate may be very different from that of a stable analogue. For citrate synthase, triosephosphate isomerase, and other enolizing enzymes, the transition state has been speculated to possess a short hydrogen bond between the enolate and the imidazole,

which may have matched pK_a values. On the other hand, the inhibitor forms a short hydrogen bond between its carboxylate oxygen and the active site base, which more closely matches its pK_a value, as illustrated by this work and previous crystallographic studies. Thus the hydrogen bonding interactions in the inhibitor complex and those of the reaction intermediate or transition state complex are very different due to the very large difference in the pK_a values for the conjugate acids of the inhibitor vs the enolate of acetyl-CoA. Furthermore the time scale for the two situations is completely different, with the inhibitor binding constant corresponding to a relaxed, solvated species probed on a very long time scale while the reactive intermediate or transition state might correspond to a structure that is a substantial excursion from relaxed coordinates. In these respects, the inhibitors might provide a poor view of the reaction coordinate.

The data reported here support a short hydrogen bond between the carboxyl group of CMX and Asp 375 of citrate synthase, in agreement with X-ray results. For proton abstraction from acetyl-CoA, the carboxylate oxygen of Asp 375 is expected to approach in an orientation perpendicular to the plane defined by the acetylthio group. In contrast, optimal hydrogen bond formation to CMX and related acetyl-CoA analogues would result from in-plane approach. The short hydrogen bond distance could result from rotation of the inhibitor toward the Asp 375 carboxylate to optimize the angle for hydrogen bonding while sacrificing optimal distance.

In conclusion, this paper presents an NMR study of an unusually short hydrogen bond in a citrate synthase−inhibitor complex, previously identified by X-ray crystallography. These NMR studies provide three main conclusions as follows:

1. The ^{13}C chemical shift tensor elements of 233 ± 4, 206 ± 5 and 105 ± 2 ppm indicate that the carboxyl group of the inhibitor is deprotonated when bound to citrate synthase.

2. The proton chemical shift of 22 ± 5 ppm and the heteronuclear correlation peak volumes measured for wild-type and mutant enzymes complexes with labeled inhibitor indicate a very short hydrogen bond between the carboxyl group of CMX and the carboxyl group of Asp 375, confirming the results of a previous X-ray crystallography study (*1*).

3. Syn and anti hydrogen bonded carboxylates apparently exhibit distinct ^{13}C NMR chemical shift signatures, the syn structure being more influenced by hydrogen bonding.

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