Proton Uptake Accompanies Formation of the Ternary Complex of Citrate Synthase, Oxaloacetate, and the Transition-State Analog Inhibitor, Carboxymethyl-CoA. Evidence That a Neutral Enol Is the Activated Form of Acetyl-CoA in the Citrate Synthase Reaction†

Linda C. Kurz,*,‡ Saurabh Shah,‡ Brian R. Crane,§ Lynda J. Donald,§ Harry W. Duckworth,§ 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, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2

Received March 11, 1992; Revised Manuscript Received June 9, 1992

ABSTRACT: Citrate synthase complexes with the transition-state analog inhibitor, carboxymethyl-CoA (CM-CoA), are believed to mimic those with the activated form of acetyl-CoA. The X-ray structure [Karpusas, M., Branchaud, B., & Remington, S. J. (1990) *Biochemistry 29*, 2213] of the ternary complex of the enzyme, oxaloacetate, and CMCoA has been used as the basis for a proposal that a neutral enol of acetyl-CoA is that activated form. Since the inhibitor carboxyl has a pK_a of 3.90, analogy with an enolic acetyl-CoA intermediate leads to the prediction that a proton should be taken up from solution upon formation of the analog complex so that the transition-state analog carboxyl is protonated when bound. We have obtained evidence in solution for this proposal by comparing the isoelectric points and the pH dependence of the dissociation constants of the ternary complexes of the pig heart enzyme with the neutral ground-state analog inhibitor, acetonyl-CoA (KCoA), and the anionic transition-state analog inhibitor (CMCoA) and by studying the NMR spectra of the transition-state analog complexes of allosteric (*Escherichia coli*) and nonallosteric (pig heart) enzymes. The pH dependence of the dissociation constant of the ground-state analog indicates no proton uptake, while that for the transition-state analog indicates that 0.55 ± 0.04 proton is taken up when the analog binds to the citrate synthase–oxaloacetate binary complex. The overall charges of ternary complexes of the pig heart enzyme with the transition-state and ground-state analog inhibitors are the same, as monitored by their isoelectric points. In [1-^{13}C]carboxymethyl-CoA binary complexes and in the oxaloacetate ternary complex of the allosteric enzyme in the absence of KCl, the chemical shift of the bound CMCoA carboxylate indicates that the inhibitor is bound as the anion. In ternary complexes with OAA (or with OAA plus KCl for the allosteric enzyme), the chemical shift of the bound CMCoA carboxyl is consistent with inhibitor carboxyl protonation in the active site. The chemical shift of the bound carboxyl of CMCoA is unaffected by pH (pH 6.4–10.0), indicating that the potential ionization of the bound carboxyl is suppressed as long as the enzyme ternary complex remains intact. The allosteric enzyme from *E. coli* requires KCl to achieve protonation of CMCoA, implying that one effect of KCl is to facilitate the activation, as well as the binding, of acetyl-CoA.

Citrate synthase (EC 4.1.3.7) catalyzes the Claisen condensation of oxaloacetate (OAA)[1] with acetyl-CoA to form citrate. In analogy with the mechanism of nonenzymatic Claisen condensations of oxygen esters, an α-carbanion has been widely accepted as a discrete intermediate in the enzymatic Claisen condensations of thioesters (Walsh, 1977). The presence of a carbanion intermediate has been inferred from three observations: (1) Proton exchange between the α-methyl hydrogens of acetyl-CoA and solvent occurs in ternary complexes with some analogs of OAA (*S*-malate; Eggerer, 1965) and in ternary complexes of OAA with poor thioester substrates (propionyl-CoA; Weidman & Drysdale, 1979, acetonyl-CoA; this work). (2) An intermediate, presumed to be an enethiolate, has been kinetically detected during turnover with the slow substrate acetyldithio-CoA (thioxoacetyl-CoA; Wlassics & Anderson, 1989). (3) Carboxymethyl-CoA (CMCoA), a structural analog of enolic acetyl-CoA ("transition-state analog inhibitor"), has a high affinity ($K_i \sim 0.02$ μM) for the enzyme in the presence of OAA (Bayer et al., 1981; this work), presumably forming a close analog of the activated intermediate complex in which one oxygen of the inhibitor is the analog of the methylene carbon and the other is the analog of the hydroxyl of acetyl-CoA (Figure 1). Generation of a significant concentration of the carbanion at neutral pH is difficult; the pK_a of acetyl-CoA is estimated to be ~20 (Fedor & Gray, 1976). None of the data obtained previously clearly indicate the actual charge state of the intermediate. The X-ray structure of the ternary complex of OAA and CMCoA (Figure 1; Karpusas et al., 1990) suggests how the enzyme might generate a reactive acetyl-CoA species with all of the observed properties while avoiding the high cost of generating a carbanion bearing a full negative charge. One of the carboxy oxygens of CMCoA is

† Supported by National Institutes of Health Grant GM33851 (L.C.K., G.R.D.) and the Natural Sciences and Engineering Research Council of Canada (H.W.D.). The Washington University High Resolution NMR Service Facility is funded in part through NIH Biomedical Research Support Shared Instrument Grant 1 S10 RR02004 and a gift from the Monsanto Co.

‡ Washington University.

§ University of Manitoba.

[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; CD, circular dichroism; CMCoA, carboxymethyl-coenzyme A; CoA; coenzyme A; CS, citrate synthase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); KCoA, acetonyl-coenzyme A; OAA, oxaloacetate.

0006-2960/92/0431-7899$03.00/0

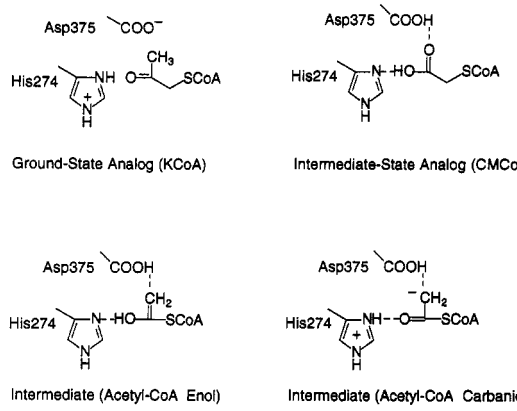

FIGURE 1: Active site cartoon of citrate synthase showing structures of bound ground-state (KCoA) and transition-state (CMCoA) analogs in comparison with that of the proposed intermediate, acetyl-CoA enol.

in hydrogen-bonding distance to Asp375[2] and the other to His274. Acetyl-CoA can be modeled into this structure to suggest that while Asp375 abstracts a proton from the methyl group of acetyl-CoA, His274 simultaneously donates a proton to the carbonyl, generating a neutral enol. New crystal structures of acetyl-CoA itself in ternary complexes with OAA analogs are consistent with this proposal (Karpusas et al., 1991). Since the CMCoA carboxyl exists as an anion at neutral pH (pK_a = 3.90) while its complex with the enzyme is an analog of the reactive intermediate, a neutral enolic acetyl-CoA intermediate leads to the prediction that a proton will be taken up from solution upon formation of the ternary OAA transition-state analog complex. This prediction is supported by the observations reported here.

MATERIALS AND METHODS

Enzymes. Crystalline citrate synthase from pig heart was a product of Sigma Chemical Co., St. Louis, MO. The citrate synthase from *Escherichia coli* was prepared as previously described (Anderson & Duckworth, 1988). After extensive dialysis against a standard buffer (see Table I), enzyme samples were concentrated to ~250 mg/mL by centrifugation in a CF50 Centriflo concentrator (Amicon Corp.). The [2-^{13}C]-oxaloacetate citrate synthase (pig heart enzyme) complex was prepared as previously described (Kurz et al., 1985).

Chemical Synthesis of [2-^{13}C]OAA. For these experiments, a chemical synthesis was used to prepare [2-^{13}C]OAA. α-Ketoglutarate, the major contaminant in our previous enzymatic synthesis of labeled OAAs (Kurz et al., 1985), inhibits the *E. coli* enzyme, competitively with OAA. β-Methyl [2-^{13}C]aspartate was prepared from [2-^{13}C]aspartate (Merck Isotopes) and methyl acetate according to the method of Biernat et al. (1961). The trifluoromethyloxazalone was prepared according to the procedure of Weygand et al. (1962) with a yield of 56%. Hydrolysis of the oxazalone to yield OAA gave a poor but adequate yield (23%). Four milliliters of ice-cold 1 N NaOH was stirred with 700 μmol of oxazalone. Production of OAA was monitored enzymatically by a modification of the DTNB method (Srere, 1969) and reached a maximum of 60–70 μmol/mL in about 2 h, after which time it declined slowly. The reaction mixture was rapidly acidified with 180 μL of ice-cold concentrated H_2SO_4, and OAA free acid was extracted into ethyl acetate. The ethyl acetate layer was evaporated, and the yellow residue was dissolved in 1 mL of ice-cold 1 N HCl. The resulting solution was stored frozen at liquid N_2 temperatures, where it was stable indefinitely. The OAA was neutralized for experiments by rapid mixing of a single addition of an amount of a 1 M Tris base solution sufficient to bring the pH to 7–8. The major contaminant resonance in the ^{13}C spectra of these OAA preparations has a ^{13}C chemical shift of 92 ppm and probably arises from a Michael addition product generated during the alkaline hydrolysis of the oxazalone. However, neither this contaminant nor the several others present in minor amounts seem to bind to the enzyme and therefore do not interfere with our experiments.

CoA Analogs. CMCoA was prepared according to the method of Bayer et al. (1981) and as previously described (Kurz & Drysdale, 1987). [1-^{13}C]CMCoA was prepared in the same way except that [1-^{13}C]iodoacetate (Merck Isotopes) was used. KCoA was prepared according to the procedure of Rubenstein and Dryer (1980).

Circular Dichroism and K_d Determinations. Spectra were collected at 20 °C using a Jasco J600 spectropolarimeter. Cell path lengths were chosen according to the objectives of individual experiments, and exact conditions will be noted under Results.

Circular dichroism titrations were performed at the single wavelength of 260 nm. Each point was time-averaged over 720 s to increase signal/noise. The data were analyzed by nonlinear regression to the general binding equation of a ligand to a single site in which a physical property ($\Delta\Theta$ in the present case) is proportional to the concentration of each species. The ordinate, delta theta, is calculated according to

$$-\Delta\Theta_{obs} = \Theta_{obs} - \Theta_L - \Theta_{CS} \quad (1)$$

where Θ_{obs} is the observed ellipticity in millidegrees (dilution corrected), Θ_L is the ellipticity of the ligand which would be observed at the concentration on the abscissa, and Θ_{CS} is the ellipticity of CS which would be observed in the absence of ligand. The data were fitted by nonlinear regression to

$$-\Delta\Theta_{obs} = a([L] + [CS] + K_d - (([L] + [CS] + K_d)^2 - 4[CS][L])^{1/2}) \quad (2)$$

where [L] is the total added concentration of ligand (CMCoA in Figure 3) and [CS] is the concentration of citrate synthase active sites. The parameter, a, is given by

$$a = -0.5(\Theta^\circ_{CMPLX} - \Theta^\circ_{CS} - \Theta^\circ_L) \quad (3)$$

where Θ°_{CMPLX} is the ellipticity (10-mm cell path length at 260 nm) of 1 μM complex, Θ°_{CS} is the ellipticity of 1 μM (uncomplexed) enzyme, and Θ°_L is the ellipticity of 1 μM (uncomplexed) ligand.

Isoelectric Point Measurements. p*I* measurements were made using a PhastGel system (Pharmacia) for isoelectric focusing with precast pH 5–8 gels. To ensure that bands had reached their final positions on the gel, each sample was run on two gels with sample application either from both ends or from only one end of the gel. The two gels were indistinguishable in all cases. This is particularly important for samples which give a very wide p*I* band.

Proton-Exchange Rate of KCoA. Experiments to measure the rate of proton exchange of the methyl protons of KCoA were performed by monitoring the disappearance of the KCoA methyl proton resonances in the 300-MHz ^{1}H NMR spectrum. The ratio of the integrated intensity of the ketone methyl proton resonance at 2.3 ppm to that of one of the pantothenate methyls at 0.9 ppm was recorded as a function of time. Exchange mixtures contained 5 μmol of KCoA, 1 mg of CS, 0.35 μmol of OAA in 0.7 mL of D_2O buffer, and 50 mM

[2] Numbering of amino acid residues is that of the pig heart enzyme.

potassium phosphate, pD 7.5. Controls contained 5 μmol of KCoA ± 1 mg of CS in the same buffer.

Carbon NMR. Carbon-13 spectra were obtained at 125.7 MHz by using a Varian VXR-500 spectrometer equipped with a 5-mm multinuclear probe. The pulse width was 21°, and the recycle time was 0.47 s. Proton-decoupled spectra were obtained using Waltz decoupling. The temperature of the sample was 10 °C. The final sample composition included 25% D_2O (for internal lock) and 0.15 M acetonitrile (as internal chemical shift standard). The cyano resonance of the standard was assigned the value of 118.9 ppm. Appropriate amounts of 0.1 N NaOD or 0.1 N DCl were added to change the pH* (the superscript indicates pH meter reading) of samples. The pH* of samples was checked before and after the NMR experiment. No attempt was made to correct the pH reading for the D_2O content of the sample.

RESULTS

High-Wavelength CD of CS and Determination of K_d for CoA Analogs. The high-wavelength CD spectrum of CS (250–300 nm) shows two peaks with positive ellipticity centered around 295 and 260 nm with a negative ellipticity peak centered around 280 nm (Figure 2). The spectrum is sensitive to complex formation with ligands. Binary complex formation with OAA results in minor changes; the ellipticity in the 260- and 280-nm bands becomes more positive, while the 295-nm band is largely unaffected. Larger changes in the CD spectrum occur on complex formation with CoA analogs which are mainly characteristic of the binary vs ternary nature of the complex.

Binary Complexes. Complexes of CS with CoA or its derivatives show decreased ellipticity in the 260-nm region with little or no change in the 295-nm peak. At saturation with the ligand (based on K_d value, vide infra), the ellipticity at 260 nm is close to 0. This pattern is quantitatively the same for binary complexes with acetyl-CoA and KCoA as it is for CMCoA. Typical binary complex spectra illustrated by those obtained with CMCoA are shown in panel A of Figure 2.

Ternary Complexes. The CD changes for ternary complexes occur in the same region but are over 2-fold greater in magnitude at 260 nm than those for binary complexes. Smaller changes occur in other regions (280 nm). Spectra for CS–OAA–CoA and CS–OAA–CMCoA are shown in panel B of Figure 2. As a consequence of the enzyme's high affinity for OAA ($K_d \sim 1$ μM; Alter et al., 1990), OAA concentrations in these experiments 3 times those of enzyme assure OAA saturation. Owing to the comparatively high dissociation constant of CoA from its complex with CS–OAA, the spectrum shown in panel B was acquired using a short path cell (1 mm) and ligand and enzyme concentrations sufficiently high to ensure saturation of sites. Titration data obtained at lower concentrations and analyzed as described below yielded a K_d of 14 ± 1 μM. Fractional saturations given in the figure were calculated from the values of the K_ds (although little correction is required since the spectra were collected under conditions close to stoichiometric binding).

K_d Determinations. These CD changes have been used to determine the binary complex dissociation constant of the enzyme with CoA analogs. Data taken at 260 nm, plotted as a difference-spectrum titration curve, are shown in panel A of Figure 3 for CS and CMCoA. For the binary CMCoA–CS titration shown in the figure, nonlinear regression analysis of the data using eq 2 yielded values of 52 ± 4 μM and 0.130

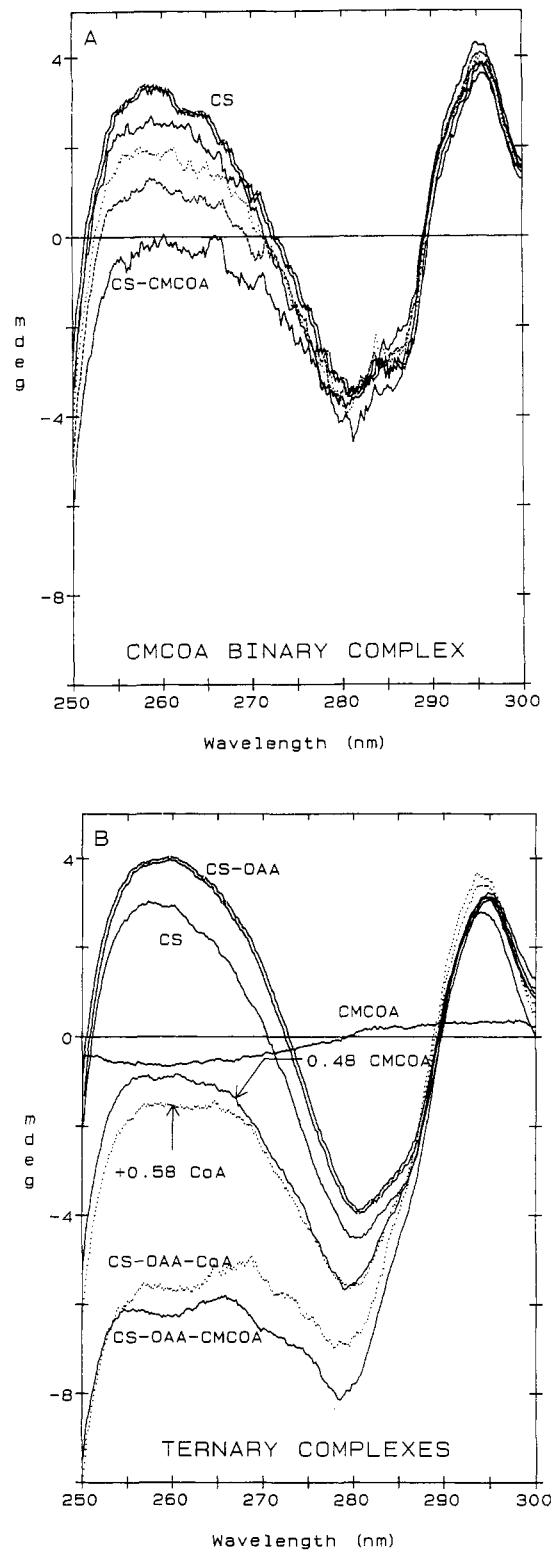

FIGURE 2: Circular dichroism spectra of citrate synthase and its complexes. (A) Binary CS–CMCoA complex. Spectra were collected from solutions in 0.1-cm cell. (Double line) 153 μM CS in 50 mM Tris-HCl, 1 mM EDTA, pH 7.5; (single line) 153 μM CS with 24 μM CMCoA; (dotted line) 153 μM CS with 68 μM CMCoA; (dashed line) 153 μM CS with 135 μM CMCoA; (dash-dotted line) 153 μM CS with 338 μM CMCoA. (B) CS–OAA binary complex and ternary complexes. Spectra were collected in 1-cm cell except for CS–OAA–CoA, which was collected in 0.1-cm cell. Curves from top left: (double line) 15.3 μM CS–OAA; (single line) 15.3 μM CS, no ligands; (single line) 15.3 μM CMCoA (typical blank for all CoA analogs); (single line) 15.3 μM CS–OAA with 0.48 fractional saturation by CMCoA; [dotted line (upper)] 153 μM CS–OAA with 0.58 fractional saturation by CoA; [dotted line (lower)] 153 μM CS–OAA–CoA; (single line) 15.3 μM CS–OAA–CMCoA.

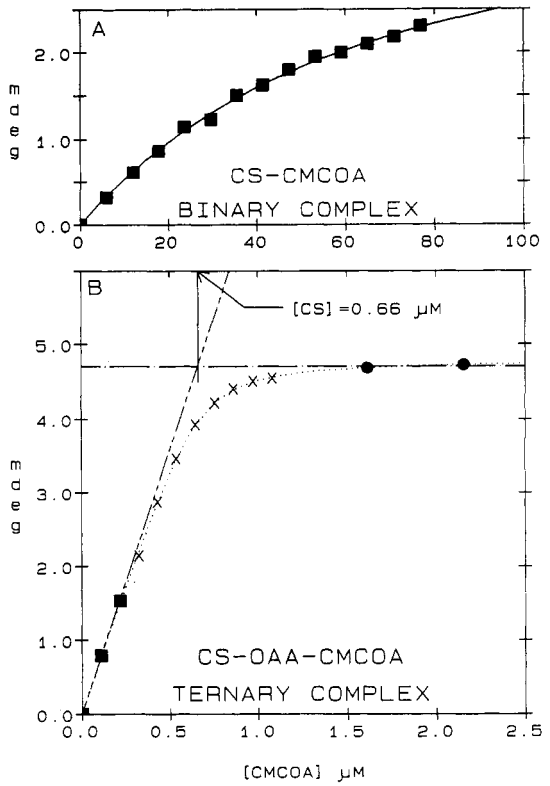

FIGURE 3: Titration curves at 260 nm for CS–CMCoA binary complex and CS–OAA–CMCoA ternary complex. (A) 15.3 μM CS titrated with CMCoA in 1-cm path length cell, 50 mM Tris-HCl, 1 mM EDTA, pH 7.5, 20 °C. Solid squares are actual data; line is calculated from nonlinear regression fit of the data to eq 2. (B) 0.66 μM CS–OAA titrated with CMCoA in 10-cm path length cell, 50 mM Tris-HCl, 1 mM EDTA, pH 7.5, 20 °C. Solid squares, ×'s, and solid circles are actual data from a single experiment representing the linear, nonlinear, and final parts of the data set, respectively. Dotted line is calculated from nonlinear regression fit of the data to eq 2. Dash-dot line is the average of the two end-point data, solid circles. Dash-dot-dot line is the linear regression fit of the first three points (stoichiometric titration data are solid squares). Solid line shows the intersection of the end-point and stoichiometric titration line.

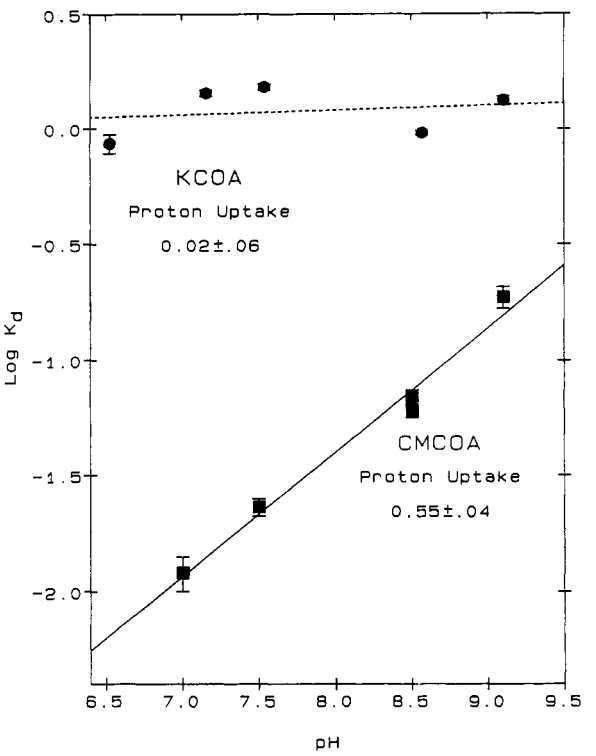

FIGURE 4: pH dependence of K_d for dissociation of transition-state analog inhibitor, CMCoA, and ground-state analog inhibitor, KCoA, from CS–OAA–inhibitor complexes. Lines (solid line for CMCoA complex and dotted line for KCoA complex) are calculated from the linear regression fit of log K_d data. Slopes of the regression lines are given as proton uptake values in the figure.

$\pm$ 0.005 mdeg μM^{-1} cm^{-1} for the parameters K_d and a, respectively.

Measurement of the value of K_d for the dissociation of KCoA from the ternary complex CS–OAA–KCoA over the desired pH range is similarly straightforward. However, reliable determination of K_d values for ternary complexes of CMCoA places great demands on the precision of the data. Very tight binding and signal size considerations combine to force use of less than optimal conditions. Panel B of Figure 3 shows the titration data obtained at pH 7.5. The curved line is calculated from the results of a nonlinear fit of the data to eq 2 in which the values of three parameters (a = 3.63 $\pm$ 0.04 mdeg μM^{-1} cm^{-1}, [CS] = 0.66 $\pm$ 0.01 μM, and K_d = 0.023 $\pm$ 0.002 μM) were determined from the regression. This value of [CS], 0.66 μM, can also be obtained from the value of the abscissa at the intersection of the lines drawn through the initial and final data points, the two intersecting lines representing a stoichiometric titration, and is equal to the value calculated independently from the absorbance at 280 nm (ϵ_{280} = 8.72 × 10^{-2} mL μM^{-1}). The high quality of the data for this pH is illustrated by the agreement between these three values for [CS]. Data obtained at higher pH are of lower quality because of the increasing instability of the free enzyme. At lower pH, the K_d value is more difficult to measure because of the higher affinity of the inhibitor. As illustrated by the data in panel B of Figure 3, the K_d value is determined by the deviation of the experimental points from the two intersecting lines representing a stoichiometric titration. At pH 6.5 (data not shown), there is no usable deviation of the experimental data from the stoichiometric titration. At pHs other than 7.50, we used direct measurements of [CS] (from the 280-nm absorbance of the solution) and a (from independent measurements of the ellipticity extent at saturation with ligands) so that K_d was the only unknown parameter. With sufficient precision and additional controls, it is possible to obtain K_d values using data surprisingly close to the region of the stoichiometric titration.

The pH dependences for the dissociation constants of KCoA and CMCoA from their respective ternary complexes are shown in Figure 4. The pH dependence of the log of the dissociation constant of CMCoA from its ternary complex has a positive slope of 0.55 $\pm$ 0.04. In contrast, the dissociation constant of KCoA, the structurally related but neutral ligand (which undergoes the CS-catalyzed solvent exchange of its methyl protons, vide infra), shows *no* pH dependence in the region studied. The slope of a plot of log K_d of KCoA vs pH has a slope of 0.02 $\pm$ 0.06. Thus, 0.55 proton equivalent is taken up on formation of CS–OAA–CMCoA. None are taken up on formation of CS–OAA–KCoA.

CS-Catalyzed Exchange of the Methyl Protons of KCoA with Solvent Protons. Over the time required for the measurement, no significant CS-catalyzed exchange of the methyl protons of KCoA occurs in the absence of OAA. In the presence of OAA–CS, the methyl protons of KCoA exchange with solvent with a rate constant of 0.03 μmol min^{-1} mg^{-1}. For the more acidic methylene protons adjacent to sulfur, however, uncatalyzed exchange is complete within 20 min.

For comparison, the CS–OAA-catalyzed exchange of the (faster) methylene proton of propionyl-CoA occurs with a

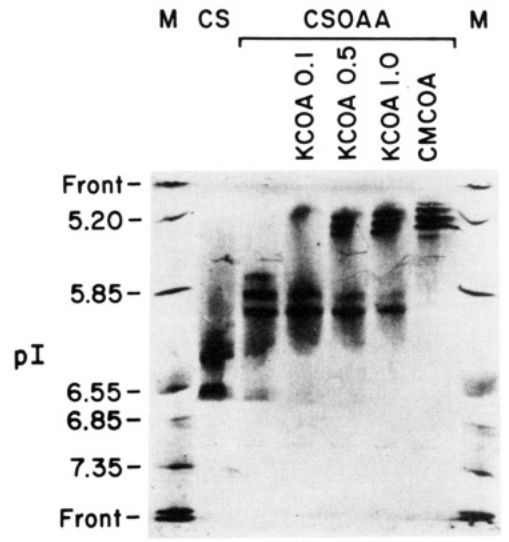

FIGURE 5: Isoelectric focusing gel comparing CS, CS–OAA, and complexes of CS–OAA with KCoA and CMCoA. [Lane 1 (left)] p*I* markers; (lane 2) CS, no ligands; (lane 3) CS–OAA binary complex, 100 μM OAA; (lane 4) CS–OAA–KCoA ternary complex, 100 μM KCoA; (lane 5) CS–OAA–KCoA ternary complex, 500 μM KCoA; (lane 6) CS–OAA–KCoA ternary complex, 1 mM KCoA; (lane 7) CS–OAA–CMCoA ternary complex, 100 μM CMCoA; (lane 8) p*I* markers.

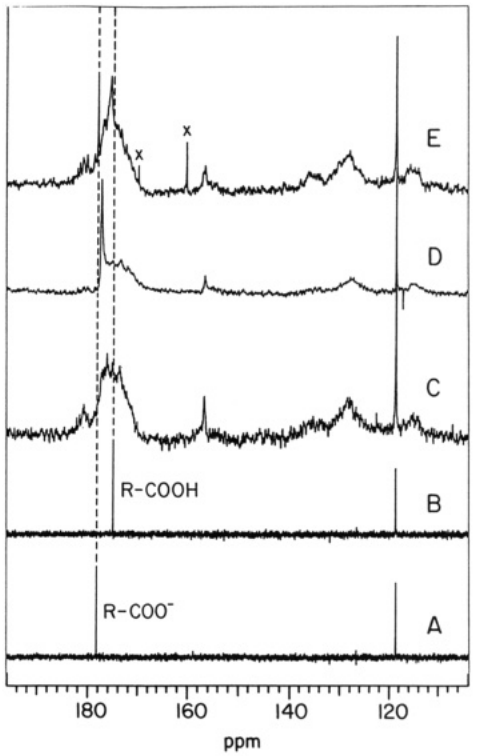

FIGURE 6: ^{13}C NMR spectra of free carboxymethyl-CoA and carboxymethyl-CoA bound to pig heart citrate synthase (all samples contain 25% D_2O, 1 mM EDTA, and 150 mM MeCN). The enzyme samples contain 50 mM Tris-HCl at the indicated pH*). (A) 1.25 mM [1-^{13}C]carboxymethyl-CoA, pH* 7.5, 2579 transients, 1-Hz filter; (B) 1.25 mM [1-^{13}C]carboxymethyl-CoA, pH* 2.0 (HCl), 4004 transients; (C) 2.8 mM pig heart citrate synthase, pH* 7.5, 25,893 transients, 10-Hz exponential filter; (D) 2.8 mM pig heart citrate synthase, 2.9 mM [1-^{13}C]carboxymethyl-CoA, pH* 7.5, 25 893 transients; (E) 2.8 mM pig heart citrate synthase, 2.9 mM [1-^{13}C]-carboxymethyl-CoA, 20 mM OAA, pH* 7.5, 72 163 transients. ×'s in spectrum E are decoupler artifacts. The protein spectra have been scaled differently to fit all of them in a single figure.

rate constant of 6.6 μmol min^{-1} mg^{-1}, although the value of V_{max} with this substrate is only 0.006 μmol min^{-1} mg^{-1} (Weidman & Drysdale, 1979). With acetyl-CoA, the value of V_{max} is 150–200 μmol min^{-1} mg^{-1}.

Careful inspection of ^{13}C NMR spectra of a 1 mM CS–[2-^{13}C]OAA–KCoA solution obtained over 9 h shows no evidence of new resonances, and the [2-^{13}C]OAA resonance observed in the CS–OAA binary complex is not significantly diminished in intensity in the CS–OAA–KCoA complex. Therefore, it is unlikely that a condensation reaction occurs with this analog.

Isoelectric Point of CS and Its Complexes. In an isoelectric focusing experiment, the electric field would separate the enzyme from most loosely bound ligands. Complexes of CS with very tightly bound ligands migrate intact with characteristic p*I*s (Figure 5). As the enzyme forms tight complexes with its anionic ligands, the p*I* drops and the pattern sharpens. The free enzyme (lane 2 of Figure 5) has a very broad p*I* from 6.1 to 6.4 with ill-defined bands at 6.6 and 6.3. The pattern given by the OAA–CS complex (lane 3) is sharper, with two main bands at 5.9 and 6.0 and a blur extending from 5.7 to 6.1. The sharpest patterns are given by ternary complexes. The CS–OAA–CMCoA (lane 7) and the CS–OAA–KCoA (lane 6) complexes (the latter at sufficient concentration to maintain complex integrity) show nearly identical patterns with three main bands at 5.25, 5.18, and 5.08.

^{13}C NMR of CMCoA Carboxyl and Its Complexes with Nonallosteric (Pig Heart) CS. The free anion of CMCoA (spectrum A of Figure 6) resonates at 178.1 ppm. The protonated form (spectrum B) resonates at 174.9 ppm. The total protonation shift is $\Delta\delta = -3.2$. This value is typical of aliphatic carboxylates (Jardetzky & Roberts, 1981).[3] Regression analysis of chemical shift data obtained from solutions of intermediate pH as a function of pH yields a value of $pK_a = 3.90 \pm 0.01$ for the acid dissociation constant of the carboxyl of CMCoA.

The interpretation of NMR spectra of enzyme-bound ligands requires knowledge of their dissociation constants. The requisite data are summarized in Table I for both the allosteric and nonallosteric enzymes. For ternary complexes, all of the ligand is bound at the millimolar concentrations of CS used in the NMR experiments unless the concentration of ligand exceeds that of binding sites.

The spectrum[4] of the enzyme (pig heart) in the absence of any ligand is shown in spectrum C of Figure 6. The binary complex spectrum with carboxymethyl-CoA is shown in spectrum D. The chemical shift of the carboxyl carbon in the binary complex lies 0.7 ppm below the anion resonance of the free ligand. The resonance is substantially broadened as is typical of bound ligands. The ternary complex spectrum (with

[3] The carboxyl resonance of [1-^{13}C]CMCoA samples prepared in the absence of EDTA is broadened almost beyond detection presumably by adventitious paramagnetic ions in the solution. A carboxylate oxygen, the CoA sulfur, and a metal cation could form a five-membered ring, and thus CMCoA may have significant chelating properties. To prevent this paramagnetic broadening, all samples routinely contained 1 mM EDTA. Most samples of CMCoA contain a small amount of a substance with a ^{13}C resonance at 178.1 ppm which does not bind to the enzyme. In addition to the sulfhydryl, CoA contains other nucleophilic sites such as the 3′-phosphate, and during CMCoA synthesis, attack of iodoacetic acid on these other sites could produce a doubly labeled CoA. The ^{31}P and ^{1}H spectra of most samples show minor additional resonances. The presence of these contaminants does not affect any of our conclusions.

[4] The signal to noise ratio in these spectra varies primarily because of the differing numbers of transients collected. The contaminant peak at 178 ppm shows significant variation in its intensity over the 6.4–10.1 pH range examined.

Table I: K_d and Chemical Shifts for [1-^{13}C]Carboxymethyl-CoA Bound to Allosteric[a] and Nonallosteric[b] Citrate Synthases

conditions	enzyme	K_d (μM)	chem shift[c] (ppm)
Tris standard buffer[d]	none		178.1
pH 2 (HCl), 1 mM EDTA	none		174.9
buffer	nonallosteric	51 ± 2[e]	177.4
buffer + OAA		0.023 ± 0.002[e]	175.2
buffer + KCl	allosteric	180 ± 40[f]	178.0
buffer, no additions		325 ± 75[f]	178.1
buffer + OAA + KCl		3.98 ± 0.39[f]	175.2
buffer + OAA, no KCl		$[L]_{0.5}$ = 26 ± 4[f,g]	177.4
previous sample + KCl		(not measured)	175.3

[a] *E. coli* enzyme (hexamer in presence of KCl, 300 000 Da; mostly hexamer in absence of KCl (Tong & Duckworth, 1975). [b] Pig heart enzyme (dimer, 100 000 Da). [c] Chemical shift values obtained in replicate experiments vary by ≤0.2 ppm. [d] 20 mM Tris-HCl, 1 mM EDTA, pH 7.8, for *E. coli* enzyme; 50 mM Tris-HCl, 1 mM EDTA, pH 7.5, for pig heart enzyme. Final concentrations of additions were 0.1 M for KCl, 20 mM for OAA. [e] K_d determined by CD titration. [f] K_d determined by ANS displacement (Talgoy et al., 1979). Hyperbolic saturation was observed. [g] Sigmoid saturation was observed, so only the value of the ligand concentration at half-saturation, $[L]_{0.5}$, is given.

unlabeled OAA) is shown in spectrum E. The carboxyl carbon chemical shift lies 2.9 ppm below the anion resonance at a value very close to that of the protonated carboxyl.

^{13}C NMR of CMCoA Carboxyl and Its Complexes with Allosteric (E. coli) CS. The spectra from the allosteric CS (*E. coli*) are essentially the same as those obtained from the nonallosteric enzyme, provided that KCl is present, a medium in which the *E. coli* enzyme has its highest activity (Faloona & Srere, 1969). The only difference noted were a larger line width and lower signal to noise ratio, an expected consequence of binding to a larger (280 000 Da) protein. In the absence of KCl, however, the major resonance of the ternary complex is unshifted from that of the binary, indicating binding of CMCoA as the anion (even in the presence of 20 equivalents of OAA). Upon the addition of KCl, a spectrum with the primary resonance at 175 ppm is obtained which is identical with one in which KCl had been present throughout.

The chemical shifts for CMCoA bound to both the nonallosteric (pig heart) and allosteric (*E. coli*) enzymes under various conditions are summarized in Table I.

The chemical shift of the bound CMCoA suggests that its carboxyl is protonated in the active site while the pH of the bulk solution is many pH units above its pK_a. We have investigated the pH dependence of the bound resonance over the pH range 6.4–10.1. For any experiment at pH extremes, it is necessary to determine whether the ternary complex remains intact so that any changes in chemical shifts or relative peak heights can be properly interpreted in terms of changes in the bound ligand. While dissociation constant data for OAA are not available over a wide pH range, the characteristic, large chemical shift change in the 2-^{13}C of OAA, $\Delta\delta$ = +6.8, which occurs upon formation of the ternary complex with carboxymethyl-CoA (Kurz et al., 1985), can be used to monitor the integrity of the ternary complex. If OAA dissociates, a sharp resonance will appear at the position of the free OAA, ~200 ppm, or if decarboxylation occurs (low pH), a sharp resonance will appear at the position of pyruvate, ~204 ppm. If carboxymethyl-CoA dissociates, the OAA binary complex resonates at 204.3 ppm.

In the experiment shown in Figure 7, [1-^{13}C]carboxymethyl-CoA was added to a binary complex of the enzyme with [2-^{13}C]-OAA. Spectrum A is that of a sample in which a substoichiometric amount of [1-^{13}C]carboxymethyl-CoA has been

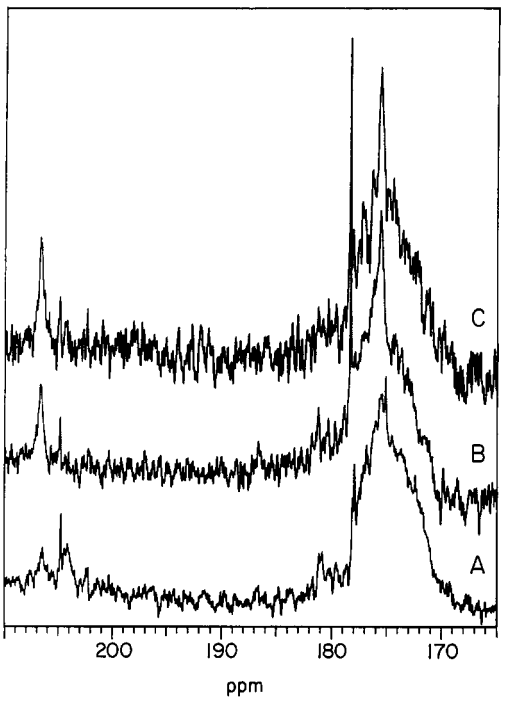

FIGURE 7: ^{13}C NMR spectra of [2-^{13}C]OAA and [1-^{13}C]carboxymethyl-CoA bound to pig heart citrate synthase. (A) 3.0 mM citrate synthase, 3 mM [2-^{13}C]OAA, 1 mM [1-^{13}C]carboxymethyl-CoA, pH* 7.5, 55 302 transients; (B) 3.0 mM citrate synthase, 3 mM [2-^{13}C]OAA, 3 mM [1-^{13}C]carboxymethyl-CoA, pH* 6.4, 97 783 transients; (C) 3.0 mM citrate synthase, 3 mM [2-^{13}C]OAA, 3 mM [1-^{13}C]carboxymethyl-CoA, pH* 10.1, 43 114 transients.

added to a solution of the binary complex of citrate synthase with [2-^{13}C]OAA. Resonances of OAA ternary complex, OAA binary complex, and carboxymethyl-CoA ternary complex are visible at 206.6, 204.1, and 175.2 ppm, respectively. The sharp peak at 204.7 ppm is pyruvate, and that at ~178 ppm is a contaminant of carboxymethyl-CoA. Spectra B and C were obtained after a full equivalent of carboxymethyl-CoA (at pH 7.5, spectrum not shown) was added and the pH was adjusted to either 6.4 or 10.1, respectively. These spectra are essentially identical, indicating that the complex neither titrates nor dissociates over the pH range examined.[3] Even at pH 5, where the enzyme complex is insufficiently soluble to obtain a spectrum of the bound ligands, no free ligands or their decomposition products are released into the solution (as indicated by the absence of NMR signals from free ligands or decomposition products in the supernatant).

DISCUSSION

Three results reported here support the proposal that the transition-state analog inhibitor, CMCoA, is protonated when bound to citrate synthase: (1) Proton uptake from solution occurs when the complex forms. (2) The p*I* of the CMCoA complex is the same as that of the neutral-ligand KCoA complex. (3) The values of the ^{13}C chemical shift of the CMCoA carboxyl bound into ternary complexes with both allosteric and nonallosteric enzymes is consistent with protonation, while the values in binary complexes indicate an anion.

KCoA Is a Ground-State Analog. Many of the arguments in this section rely upon the behavior of *analogs* of mechanistically significant states. The data supporting the CMCoA ternary complex as an analog of a reactive acetyl-CoA intermediate state have been presented elsewhere (Bayer et al., 1981; Karpusas et al., 1991). We have chosen the KCoA

ternary complex as an analog of the acetyl-CoA ground-state complex. While it is apparent from the structure of KCoA that this analogy must have some validity, it is useful to explore its extent. The absence of a CS-catalyzed OAA condensation product does not exclude the possibility of KCoA participation in early parts of the catalytic cycle. When bound into certain ternary complexes, the reactive carbon of acyl-CoA substrate analogs can manifest increased acidity of their attached protons by a significant CS-catalyzed exchange with solvent protons. While slower than observed with thioester substrate analogs, the rate of CS-catalyzed exchange of the methyl protons of KCoA indicates the enzyme's ability to activate the methyl carbon for condensation. The extra methylene group in KCoA may prevent any condensation with OAA from actually occurring as a result of steric interference with the proper positioning of the two substrates. Actually, KCoA was chosen for comparison with CMCoA because both analogs have a methylene group not present either in the ground state or in the activated intermediate state of the natural substrate, acetyl-CoA.

Origin of the CD Changes. The largest changes we observe occur in the region of the CoA adenine absorbance. This relatively intense near-UV transition is subject to μ–μ coupling with protein aromatic residues intensifying its very small intrinsic CD signal. Intensification of protein CD probably arising by the same mechanism may account for the changes at 280 nm. Induced CD in ligand absorptions (extrinsic Cotton effects) have previously been observed as a consequence of the binding of nucleotides to proteins [i.e., Kagi et al. (1971)]. It is interesting that for citrate synthase the types of changes observed appear to be characteristic of the binary or ternary nature of the CoA analog complex. The structural significance of this observation may become apparent when X-ray structures of CoA–analog binary complexes are obtained.

pH Dependence of the Formation of Ground-State and Intermediate-State Analog Complexes. The positive slope, 0.55, of the pH dependence of log K_d for dissociation of CMCoA from its ternary complex indicates that formation of that complex requires a proton. While in its simplest form a proton requirement would imply an integral stoichiometric coefficient, this is neither required nor expected for macroscopic equilibria between polyelectrolytes [i.e., Glick (1968) and Parsons and Raftery (1972)]. First, there are several residues in the active site of the enzyme which could titrate in this pH range and whose pK_as may be perturbed either through direct interaction with the ligand or as a consequence of the drastic change in environment in the closed ternary complex. The active site in the ternary complex is buried some 15 Å from contact with the bulk solution. The pyrophosphate and 3′-phosphate groups of free CMCoA certainly titrate in the pH range studied and their pK_as are also subject to perturbation upon binding. Second, there are one or more potentially pH sensitive conformational equilibria involving the free enzyme. The significant observation is that the slope of the pH dependence of log K_d of anionic CMCoA is significantly more positive than that of neutral KCoA.

The linearity of the plot of log K_d of CMCoA vs pH suggests that the values of the pK_as of the important ionizations are far removed from the studied pH range (pH 6.5–9.5). This is consistent with the participation of the carboxyl of CMCoA (pK_a = 3.90).

pIs of Ground-State and Intermediate-State Analog Complexes. In an isoelectric focusing experiment, CS does not focus in a narrow band in the absence of ligands. The existence of multiple charge forms of the enzyme may be inferred from this observation. It is tempting to speculate that these multiple forms are related to the "open" and "closed" forms observed crystallographically. Both crystal forms have been observed from the same solution (Liao et al., 1991).

Under our conditions, if a ligand binds tightly ($K_d \leq 5\ \mu M$), then the complex migrates intact in spite of any tendency for the pH gradient to separate protein and ligand. As would be expected for the binding of anionic ligands, the p*I*s of the complexes are found at more acid pH than that of the free enzyme. Thus, the OAA–CS complex has broad p*I* bands at 5.9 and 6.0, while the ternary complexes with CMCoA or KCoA have their sharp principal bands at 5.25, 5.18, and 5.08. We always observe multiple bands for each complex. Increasing age of the sample may introduce additional small satellite bands, but the observation of the main multiple bands seems to be independent of storage time. This does not rule out deamidation or another degradative process as responsible for the multiple-band pattern. KCoA does not bind sufficiently tightly to CS–OAA to migrate as a single complex in the absence of excess KCoA. When a large excess is added, however, it is clear that the ternary complex with this ligand has the same p*I* as that with CMCoA. The minimum conclusion from this observation alone is that at the p*I* of the complexes the total charges of the complexes are the same and thus a univalent cation must be bound along with the CMCoA anion. The most likely univalent cation is a proton.

[^{13}C]Carboxyl Chemical Shift in Binary and Ternary Complexes of CMCoA with Nonallosteric and Allosteric Citrate Synthases. The carbon chemical shift of the carboxylate is sensitive to its protonation state (Figure 6; Table I). In the binary complexes, only a small shift from the free anion resonance is observed and there seems little reason to question that the carboxyl remains negatively charged in that complex.

In the ternary complexes, the chemical shift change is 90% of that observed upon protonation in free solution. This result is consistent with the proposal (vide supra) that an anionic analog of a neutral intermediate should take up a proton when bound. While a protonated CMCoA carboxyl seems entirely reasonable, such a conclusion deriving solely from the value of its chemical shift would have to be viewed cautiously. The carboxyl–carboxylate chemical shifts are sensitive to the environment and particularly to the details of hydrogen bonding (Maciel & Traficante, 1960; Kurz et al., 1992). The values of carboxylate chemical shifts in low dielectric constant non-hydrogen-bonding solvents overlap the values for protonated carboxyls found in high dielectric constant hydrogen-bonding solvents. In the hydrophobic environments found in micelles, membranes, and many proteins (Cistola et al., 1987, 1989), carboxylate chemical shifts show significant shielding compared to the values found in aqueous environments.

The results with the allosteric enzyme bear upon this issue. There is considerable evidence suggesting that while catalytic residues are completely conserved between the allosteric and nonallosteric enzymes, other features of the active site of these two enzymes must differ (Bhayana & Duckworth, 1984). Overall, there is about 27% amino acid residue identity. The allosteric enzyme exists in two conformational states, and kinetic evidence shows that acetyl-CoA binds better to the active (R) than the inactive (T) state. One can shift the allosteric equilibrium in favor of the R state by addition of KCl (Faloona & Srere, 1969; Anderson et al., 1991). These conformational states may be related to the open and closed forms of the pig heart enzyme. Further, the allosteric enzyme binds substrates and transition-state analog inhibitors with

much lower affinity (Table I and unpublished results) and shows considerably less specificity for OAA analogs. α-Ketoglutarate is a reasonably good inhibitor of the allosteric enzyme (Anderson & Duckworth, 1988), while the K_d for the nonallosteric CS is in the millimolar range (Srere et al., 1973; Kurz et al., unpublished results). In some sense the active site of the allosteric enzyme is less tightly constrained. The chemical shifts for both OAA and CMCoA in ternary complexes are identical for both enzymes if 0.1 M KCl is present in the solution of the allosteric enzyme. In the absence of KCl, the chemical shift of CMCoA in the *E. coli* enzyme ternary complex is only 0.7 ppm lower than that of the binary complex. The CMCoA is probably still anionic. For the allosteric enzyme, formation of the CMCoA complex with all of the features of a transition-state analog complex requires the presence of KCl.

It is well kept in mind that the issue here is whether solution evidence supports the proposal of a neutral acetyl-CoA intermediate, not *which* hydrogen-bonding scheme, both of which contain a protonated carboxyl group with a highly abnormal pK_a, best describes the situation. See Figure 1 for this alternative. The exclusion of solvent from the active site in the ternary complex suggests that if charge neutrality is to be maintained in the active site, Asp375 would have to be protonated (with a highly abnormal pK_a) and His274 would have to be charged if CMCoA were to be bound as an anion. The alternative, that carboxymethyl-CoA is protonated and hydrogen bonded to both an anionic aspartate and cationic histidine, is reasonable and supported by the present work. Either of these schemes which preserve the neutrality of the active site scheme supports an acetyl-CoA enol intermediate.

Implications for the Mechanism of Citrate Synthase. A viable alternative to the direct examination of the structures of transition states and intermediates is the careful characterization of stable analogs of these reactive and short-lived species. For citrate synthase, a convincing case can be made that the CS–OAA–CMCoA complex is an analog of a reactive intermediate in the reaction pathway. The critical structural feature of CMCoA is its anionic carboxylate (Bayer et al., 1981). The amidocarboxymethyl-CoA inhibitor does not show exceptionally high affinity for CS–OAA. Accordingly, it was suggested (Bayer et al., 1981) that an anionic intermediate was indicated for the activated acetyl-CoA. However, the X-ray structure of CS–OAA–CMCoA led Karpusas et al. (1990) to suggest a neutral intermediate which in turn suggests that an unusual ionization state of CMCoA may be bound by CS–OAA.

Unusual ionization states of transition-state analog inhibitors have been found previously and frequently provide insight into the structure of the actual intermediate. A case quite similar to CMCoA is provided by phosphoglycollate. This analog of the enediol(ate) intermediate of triosephosphate isomerase is bound as the trianion at a pH where the dianion is the predominant species (Campbell et al., 1978). These relatively old data suggested that the intermediate is an enediolate rather than an enediol. NMR data have recently demonstrated that the reactive intermediate is an enediolate (Lodi & Knowles, 1991).

Our results would seem to imply that when carboxymethyl-CoA is bound in the active site of citrate synthase, the pK_a of its carboxyl has been shifted to higher values by more than 7 pK units. The observed affinity of the enzyme–OAA complex for the form of carboxymethyl-CoA actually bound is correspondingly underestimated. However, the inability to titrate the carboxyl even at high pH may be more a consequence of the solvent inaccessibility of the active site than an indication of the actual pK_a of the CMCoA. The kinetic barrier to admit solvent base may be too high. Indeed, the structure of the ternary complex (Karpusas et al., 1990) shows that the active site is inaccessible to solvent. Similar observations have been made with other tight complexes of enzymes with ligands in unusual protonation states (Cocco et al., 1981). Different results might be obtained if it were possible to *form* the complex at high pH. Unfortunately, the instability of the free enzyme has so far prevented this approach.

The free energy cost of stabilizing an enol intermediate would be significantly less than that for the enolate (α-carbanion). A rough estimate of this advantage may be obtained using acetone as a model. The model is appropriate since the apparent pK_a of thioesters has been estimated, $pK_a^{keto} \sim 20$ (Lienhard & Wang, 1968; Fedor & Gray, 1976), to be about the same as that for acetone. For acetone, about 11 of these pK_a units are ascribed to the ionization of the enol (Chiang et al., 1984). If the partitioning of the pK_a is similar for acetone and thioesters, then stabilization of the enol of acetyl-CoA rather than the enolate may be easier by a factor as large as 10^{11}.

On the basis of analogy with oxygen esters, an enol would be less reactive than an enolate toward condensation with the carbonyl of OAA. This may not be a serious objection for two reasons. First, facile Claisen condensations with thioesters do not require a discrete α-carbanion intermediate. Wilson and Hess (1980) showed that neutral or cationic metal complexes of thioester enolates rather than actual anionic enolates are probably the intermediates in the nonenzymatic Claisen condensations of thioesters. Second, the enzyme is not required to generate its catalytic efficiency solely by activating the acetyl-CoA because it also significantly activates the other substrate through polarization of the OAA carbonyl (Kurz et al., 1985; Kurz & Drysdale, 1987). A sufficiently reactive OAA may not require the most reactive acetyl-CoA nucleophile for efficient condensation.

The active site of citrate synthase is constructed (Karpusas et al., 1990) to carry out the proposed proton transfers efficiently. The active site residues, His274 and Asp375, are universally conserved. His274 may act as an acid, donating a proton to the carbonyl of acetyl-CoA, while Asp375 acts as a base, removing a proton from the methyl group to generate the enol form of the substrate. Mutants in which these residues have been changed into ones which cannot interact or interact differently (Evans et al., 1989) have been prepared. Their characterization (Alter et al., 1990; Kurz et al., 1992) should significantly increase our understanding of the catalytic strategy of citrate synthase.

A similar catalytic strategy is indicated for the allosteric enzyme from *E. coli*. While the affinities of this enzyme for its substrates and the intermediate analog are somewhat lower, the same degree of OAA polarization and CMCoA protonation is achieved in the ternary complex in the presence of KCl. The findings that KCl must be present before the *E. coli* enzyme interacts strongly with CMCoA and before enzyme-bound CMCoA can take up its proton certainly show that the interaction of CMCoA is much more difficult when the enzyme is not already in its R state. It is interesting that the ability of that enzyme to bind CMCoA as a transition-state analog is induced by one treatment (KCl) which is known to shift the enzyme toward its R state. The quaternary structure (Tong & Duckworth, 1975) of the allosteric enzyme is also affected by the concentration of KCl. Our results suggest that the role of KCl, at least in part, is to promote an enzyme conformation

change stabilizing a reactive intermediate, and we speculate that this is the same conformation change which plays a role in allosteric regulation of the enzyme by its in vivo effectors.

REFERENCES

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

Anderson, D. H., & Duckworth, H. W. (1988) *J. Biol. Chem. 263*, 2163.

Anderson, D. H., Donald, L. J., Jacob, M. V., & Duckworth, H. W. (1991) *Biochem. Cell. Biol. 69*, 232.

Bayer, E., Bauer, B., & Eggerer, H. (1981) *Eur. J. Biochem. 120*, 155.

Bhayana, V., & Duckworth, H. W. (1984) *Biochemistry 23*, 2900.

Biernat, J. F., Rzeszotarska, B., & Taschner, E. (1961) *Liebigs Ann. Chem. 646*, 125.

Campbell, I. D., Jones, R. B., Kiener, P. A., Richards, E., Waley, S. G., & Wolfenden, R. (1978) *Biochem. Biophys. Res. Commun. 83*, 347.

Chiang, Y., Kresge, A. J., Tang, Y. S., & Wirz, J. (1984) *J. Am. Chem. Soc. 106*, 460.

Cistola, D. P., Small, D. M., & Hamilton, J. A. (1987) *J. Biol. Chem. 262*, 10980.

Cistola, D. P., Sacchettini, J. C., Banaszak, L. J., Walsh, M. T., & Gordon, J. I. (1989) *J. Biol. Chem. 264*, 2700.

Cocco, L., Groff, J. P., Temple, C., Jr., Montgomery, J. A., London, R. E., Matwiyoff, N. A., & Blakley, R. L. (1981) *Biochemistry 20*, 3972.

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

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

Evans, C. T., Owens, D., Casazza, J. P., & Srere, P. A. (1989) *Biochem. Biophys. Res. Commun. 164*, 1437.

Faloona, G. R., & Srere, P. A. (1969) *Biochemistry 8*, 4497.

Fedor, L., & Gray, P. H. (1976) *J. Am. Chem. Soc. 98*, 783.

Glick, D. M. (1968) *Biochemistry 7*, 3391.

Jarketzky, O., & Roberts, G. C. K. (1981) *NMR in Molecular Biology*, p 284, Academic Press, New York.

Kagi, J. H. R., Ki, T.-K., & Vallee, B. L. (1971) *Biochemistry 10*, 1007.

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

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

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

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

Kurz, L. C., Drysdale, G. R., Riley, M. C., Evans, C. T., Srere, P. A. (1992) *Biochemistry* (following paper in this issue).

Liao, D.-I., Karpusas, M., & Remington, S. J. (1991) *Biochemistry 30*, 6031.

Lienhard, G. E., & Wang, T-C. (1968) *J. Am. Chem. Soc. 90*, 3781.

Lodi, P. J., & Knowles, J. R. (1991) *Biochemistry 30*, 6948.

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

Parsons, S. M., & Raftery, M. A. (1972) *Biochemistry 11*, 1633.

Remington, S., Wiegand, G., & Huber, R. (1982) *J. Mol. Biol. 158*, 111.

Rubenstein, P., & Dryer, R. (1980) *J. Biol. Chem. 255*, 5976.

Srere, P. A. (1969) *Methods Enzymol. 14*, 3.

Srere, P. A., Matsuoka, Y., & Mukherjee, A. (1973) *J. Biol. Chem. 248*, 8031.

Talgoy, M. M., & Duckworth, H. W. *Can. J. Biochem. 57*, 385.

Tong, E. K., & Duckworth, H. W. (1975) *Biochemistry 14*, 235.

Walsh, C. (1977) in *Enzymatic Reaction Mechanisms*, p 759, Freeman, San Francisco.

Weidman, S. W., & Drysdale, G. R. (1979) *Biochem. J. 177*, 169.

Weygand, F., Steglich, W., & Tanner, H. (1962) *Liebigs Ann. Chem. 658*, 128.

Wilson, G. E., Jr., & Hess, A. (1980) *J. Org. Chem. 45*, 2766.

Wlassics, I. D., & Anderson, V. E. (1989) *Biochemistry 28*, 1627.