Catalytic Strategy of Citrate Synthase: Effects of Amino Acid Changes in the Acetyl-CoA Binding Site on Transition-State Analog Inhibitor Complexes†

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ABSTRACT: Acetyl-CoA enol has been proposed as an intermediate in the citrate synthase (CS) reaction with Asp375 acting as a base, removing a proton from the methyl carbon of acetyl-CoA, and His274 acting as an acid, donating a proton to the carbonyl [Karpusas, M., Branchaud, B., & Remington, S. J. (1990) *Biochemistry 29*, 2213]. CS–oxaloacetate (OAA) complexes with the transition-state analog inhibitor, carboxymethyl-CoA (CMCoA), mimic those with acetyl-CoA enol. Asp375 and His274 interact intimately with the carboxyl of the bound inhibitor. While enzymes in which these residues have been changed to other amino acids have very low catalytic activity, we find that they retain their ability to form complexes with substrates and the transition-state analog inhibitor. In comparison with the value of the chemical shift of the protonated CMCoA carboxyl in acidic aqueous solutions or its value in the wild-type ternary complex, the values in the Asp375 mutants are unusually low. Model studies suggest that these low values result from complete absence of one hydrogen bond partner for the Gly mutant and distortions in the active site hydrogen bond systems for the Glu mutant. The high affinity of Asp375Gly–OAA for CMCoA suggests that the unfavorable proton uptake required to stabilize the CMCoA–OAA ternary complex of the wild-type enzyme [Kurz, L. C., Shah, S., Crane, B. R., Donald, L. J., Duckworth, H. W., & Drysdale, G. R. (1992) *Biochemistry* (preceding paper in this issue)] is not required by this mutant; the needed proton is most likely provided by His274. This supports the proposed role of His274 as a general acid. The surprising low catalytic activity of the Asp375Glu mutant is discussed in terms of steric hindrance to the functioning of the carboxylate as general acid–base and/or in terms of an impairment of the mutant enzyme's ability to access the closed conformational form of the enzyme in which the active site is isolated from the bulk solvent. The Asp mutants are able to achieve full activation of OAA through carbonyl polarization as assessed by the [2-^{13}C]OAA chemical shift of ternary complexes. In contrast, the His274Gly mutant is unable to polarize the OAA carbonyl in binary or ternary complexes; it may be impaired in its access to the closed conformational form, and the ^{13}C chemical shift of the bound CMCoA is consistent with a carboxylate anion in an hydrophobic environment in the active site of this mutant protein.

Substrate activation is a major catalytic strategy of citrate synthase. Structural (Remington et al., 1982) and spectroscopic (Kurz et al., 1985; Kurz & Drysdale, 1987) studies of the OAA–CS[1] complex show that the enzyme effects significant polarization of the OAA carbonyl bond, thereby increasing the positive charge on the 2-carbon and enhancing reactivity toward the condensation with the nucleophilic center of the second substrate, acetyl-CoA. CS also facilitates formation of an effective nucleophilic center in the second substrate, acetyl-CoA. While an α-carbanion/enolate intermediate was generally assumed (Walsh, 1977) to be the activated form of acetyl-CoA, solid-state (Karpusas et al., 1990, 1991) and solution-state studies (Kurz et al., 1992) now point to a neutral enol intermediate. The structures of the ternary complexes of CS–OAA–CMCoA (Karpusas et al., 1990) and of CS–acetyl-CoA–malates (Karpusas et al., 1991) suggest how the enzyme avoids the high cost of generating a carbanion bearing a full negative charge. It was proposed that while Asp375[2] abstracts a proton frrom the methyl group of acetyl-CoA, His274 simultaneously donates a proton to the carbonyl, generating a neutral enol. Single-site changes in these two residues have deleterious effects on the enzyme's activity with the large reductions mainly in the value of k_{cat}, a result characteristic of catalytic residues (Alter et al., 1990). To act as general base and acid catalysts in the way proposed, a negatively charged Asp carboxylate and a positively charged His imidazolium are the most likely (but not the only possible, vide infra) charge states for these residues in the ground-state enzyme–substrate complex. In the intermediate complex, these residues will be neutral. While we cannot observe the effects of mutations in these residues on the actual intermediate they are presumed to stabilize, we can study their effects on the enzyme's complex with an analog of that intermediate, CMCoA. In the present work we report a study of the following mutations: Asp375Glu, Asp375Gly, Asp375Asn, and His274Gly. The effects of these mutations

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[1] Abbreviations: AcCoA, acetyl-CoA; AcOH, acetic acid; CD, circular dichroism; CMCoA, carboxymethyl-coenzyme A; CoA, coenzyme A; CS, citrate synthase; HBA/HBD, hydrogen bond accepting/hydrogen bond donating; IEF, isoelectric focusing; KCoA, acetonyl-coenzyme A; KOAc, potassium acetate; MeCN, acetonitrile; MeOD, CD_3OD; OAA, oxaloacetate; TFE, trifluoroethanol.

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

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on the properties of the transition-state analog inhibitor binary and ternary complexes support the roles proposed (Karpusas et al., 1990, 1991) for these residues.

MATERIALS AND METHODS

Enzymes. Crystalline citrate synthase from pig heart was a product of Sigma Chemical Co., St. Louis, MO.

The cloned enzymes were prepared following the procedures described in Evans et al. (1988, 1989) with modifications to produce the large quantities of protein required for NMR studies. Bacteria were grown in a 200-L fermentor. The fermentation broth contained 10 g/L casein hydrolysate, 5 g/L yeast extract, 6.7 g/L NaCl, 5 g/L Na_2HPO_4, and sufficient concentrated HCl (~80 mL) to adjust the final pH to 7.2. After the broth was autoclaved, 1 g/L glucose, 50 mg/L ampicillin, 64 mg/L kanamycin, and 4 L of an overnight culture in the same medium were added to the fermentor. Media ingredients were products of Sigma. After 8–10 h of growth at 27 °C with vigorous aeration, second additions were made of 1 g/L glucose and 50 mg/L ampicillin along with antifoam. After a total of ~20 h, the broth reached a final OD_{460} from 2.0–4.0; the cells were heat shocked as previously described to induce CS production (Evans et al., 1988). The final yield of harvested cells was 2–3 kg. Cell lysis and protein purification were as previously described (Evans et al., 1989).

The final eluant containing the enzyme from affinity chromatography contains CoA and OAA. For NMR and ligand-binding studies it is necessary to remove these ligands. They cannot be removed by conventional dialysis. CoA equilibrates with the external solution only very slowly, and OAA remains tightly bound even after removal of the free excess ligand. For cloned enzymes retaining significant activity (WT and Asp375Glu), a 10% excess of acetyl-CoA was added to enable OAA turnover to citrate. Citrate and CoA bind loosely to the enzyme and can be removed by diafiltration. For cloned enzymes without significant activity (Asp375Gly and His-274Gly), a catalytic amount of WT enzyme (0.1% w/w) was added along with the acetyl-CoA to convert bound OAA to citrate. Diafiltration was done in a 50-mL Amicon stirred cell equipped with a YM 50 membrane and attached to a 800-mL reservoir of buffer (50 mM K[PO_4]/1 mM EDTA, pH 7.5). To limit enzyme denaturation, the number of pressurization–depressurization cycles was minimized. Recovery of enzyme samples from CS–OAA–CMCoA experiments was accomplished by diafiltration with 800 mL of buffer containing 0.5 M guanidine hydrochloride prior to a final diafiltration with buffer.

The cloned enzyme preparations are homogeneous by several criteria and contain only the mutations intended, and their initial kinetic characterization has been described previously (Alter et al., 1990).

After diafiltration and clarification by centrifugation, 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; Kurz et al., 1992).

CoA Analogs. CMCoA and KCoA were prepared as previously described (Kurz & Drysdale, 1987; Kurz et al., 1992).

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

Isoelectric Point Measurements. p*I* measurements were made as previously described (Kurz et al., 1992).

Carbon NMR. Protein carbon-13 spectra were obtained at 125.7 MHz by using a Varian VXR-500 spectrometer equipped with a 5- or 10-mm multinuclear probe. Other details were as described previously (Kurz et al., 1992).

Models for Environmental Effects on the Carboxyl/Carboxylate Chemical Shift. Carbon-13 spectra of [1-^{13}C]-KOAc and [1-^{13}C]AcOH (99.7% ^{13}C label in the carboxyl carbon) in various deuterated solvents were collected at 150 MHz in a Varian Unity 600 spectrometer equipped with a 5-mm multinuclear probe. Chemical shifts were internally referenced to the natural abundance carbon peak of the solvent except for the aqueous sample, which was referenced to the acetonitrile methyl carbon.

[1-^{13}C]KOAc. Stock solutions of 18-crown-6 (product of Aldrich Chemical Co., Milwaukee, WI) were made in HPLC grade methanol. The requisite amount was transferred to a Wheaton vial and the methanol evaporated under an argon stream. The appropriate amount of an aqueous KOAc stock solution was added, and the samples were lyophilized. The lyophilized samples along with other associated glassware were dried in vacuo over P_2O_5. All subsequent transfer operations were performed under an argon blanket. Chloroform, methanol, and trifluoroethanol were prepurified and dried with 200 mg of neutral alumina/g of solvent. Eight hundred microliters of solvent was added to each sample vial; the vial was sealed, and the samples were stirred for 2–3 days to effect dissolution of the solids. The samples were transferred to a dry NMR tube just before spectra were recorded.

[1-^{13}C]AcOH. Stock solutions were prepared in HPLC grade protic solvents. Appropriate amounts were added to the deuterated solvent to a final total volume of 800 μL. Since samples of deuterated methanol contain a basic impurity, the methanol samples were acidified (0.1 M methanesulfonic acid, Aldrich) just prior to the NMR run. This avoids the complete esterification of the acetic acid, which has a half-life of about 20 min.

RESULTS

Isoelectric Focusing of Mutant Enzymes and Their Complexes with OAA and CMCoA. Comparison of Wild-Type (WT) Cloned CS with Sigma Pig Heart CS. The cloned enzyme (WTCS) and that isolated from pig heart (ΣCS) have generally the same qualitative behavior on isoelectric focusing gels. The free enzymes do not focus well. The OAA complex band is shifted toward acidic pH and is considerably sharper [not shown—see Kurz et al. (1992)]. The patterns for the ternary complexes of the enzymes containing both CMCoA and OAA are shifted yet further to acidic pH and show several very sharp bands. However, there is a quantitative difference in the actual p*I* values which is most evident in the ternary complex (lanes 4 and 5 in Figure 1: ΣCS–OAA–CMCoA, pig heart CS from Sigma, and WTCS–OAA–CM-CoA, cloned CS produced from *Escherichia coli*). The ternary complex pattern for the cloned enzyme is centered around pH 5.6–5.7, while that for the enzyme from pig heart is around pH 5.3–5.4. This difference was expected and represents the presence of one additional positive charge in the cloned enzyme which is not present in that isolated from pig heart. The construct of the enzyme expressed in *E. coli* contains a five amino acid N-terminal extension which includes a histidine. There is no apparent effect of this extra sequence on any catalytic or regulatory properties of the enzymes (Evans et al., 1988).

Asp375Gly and Asp375Asn. Unlike the wild-type enzymes, neither the ligand–free enzymes (data not shown) nor the

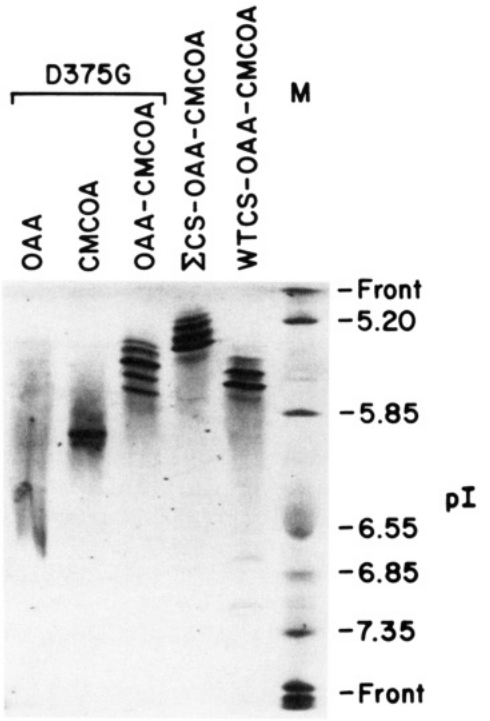

FIGURE 1: Isoelectric focusing gel comparing complexes of Asp375Gly to ternary complexes of wild-type Sigma (ΣCS) and wild-type cloned (WTCS) enzymes. [Lane 1 (left)] Asp375Gly–OAA binary complex; (lane 2) Asp375Gly–CMCoA binary complex; (lane 3) Asp375Gly–OAA–CMCoA ternary complex; (lane 4) ΣCS–OAA–CMCoA ternary complex; (lane 5) WTCS–OAA–CMCoA ternary complex; (lane 6) p*I* markers.

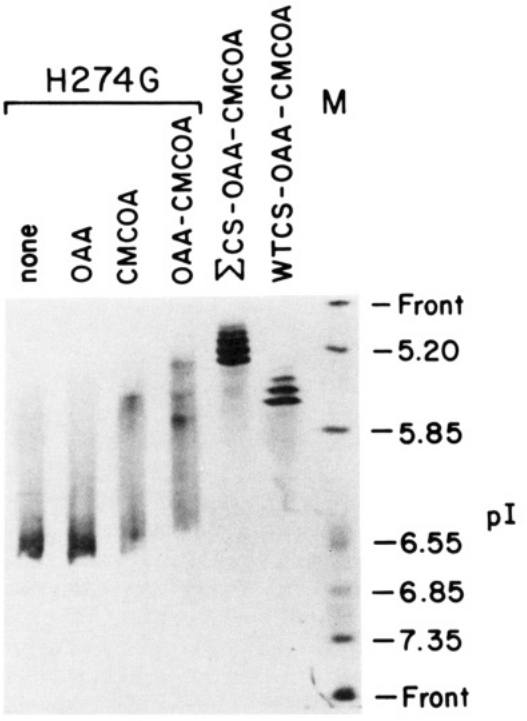

FIGURE 2: Isoelectric focusing gel comparing His274Gly and its complexes to ternary complexes of wild-type Sigma (ΣCS) and wild-type cloned (WTCS) enzymes. [Lane 1 (left)] His274Gly, no ligands; (lane 2) His274Gly–OAA binary complex; (lane 3) His274Gly–CMCoA binary complex; (lane 4) His274Gly–OAA–CMCoA ternary complex; (lane 5) ΣCS–OAA–CMCoA ternary complex; (lane 6) WTCS–OAA–CMCoA ternary complex; (lane 7) p*I* markers.

OAA complexes show any sign of focus (see lane 1 of Figure 1 for Asp375Gly–OAA). On the other hand, the CS–CMCoA *binary* complexes of Asp375Gly (lane 2 of Figure 1) and Asp375Asn (data not shown) focus in a fairly narrow band at p*I* = 6.0 unlike the wide type or any of the other mutants. Note that the center of the pattern for the ternary complex (lane 3 of Figure 1), p*I* = 5.5–5.7, is very similar to that for the WTCS (lane 5) in spite of the deletion of the chargd Asp375 residue. Data for the Asp375Asn ternary complex are quantitatively similar (not shown).

Asp375Glu and His274Gly. Neither the free enzymes nor any complex of these mutants focuses in narrow bands. His274Gly and its complexes are compared with the sharply focused ternary complexes of the wild-type enzymes in Figure 2. No defined bands are evident for the mutant. The broad pattern for the His274Gly–OAA–CMCoA ternary complex seems to extend to more acid pH than that for the free enzyme or OAA complex. Similar results are obtained with Asp375Glu (data not shown).

High-Wavelength CD of CS Mutants and K_d for CMCoA from Complexes. Qualitatively, CD spectra for mutant enzyme binary and ternary complexes resemble those for the wild-type enzymes (cloned or Sigma). The values of dissociation constants for CMCoA from ternary complexes are given in Table I. In comparison with wild-type CS–OAA, Asp375Glu–OAA and His274Gly–OAA show reduced affinity for CMCoA. [However, at the high (millimolar) active site concentrations used in NMR experiments, all of the CMCoA will be bound to the OAA complexes unless a stoichiometric excess of CMCoA over CS sites is added to the sample.] Asp375Gly has a considerably greater affinity for CMCoA than the wild-type enzyme in both binary and ternary complexes. This is evident from the shifted and sharpened IEF pattern for the binary complex shown in Figure 1. A value of K_d = 0.8 μM is obtained for dissociation of CMCoA from the binary Asp375Gly–CMCoA complex in comparison to 51 μM for the wild type. Only an upper limit ($K_d \leq 0.003$ μM)[3] can be obtained for the value of the dissociation constant of CMCoA from the mutant CS–OAA–CMCoA complex. The titration is close to stoichiometric. Asp375Asn, another neutral Asp mutant, also has increased affinity for CMCoA in comparison to the wild-type enzyme.

^{13}C Chemical Shifts of [2-^{13}C]OAA Bound to Citrate Synthase Mutants. The ^{13}C chemical shifts for the OAA carbonyl in complexes with the mutant and wild-type enzymes are given in Table I. In all cases, the cloned wild-type enzyme behaves identically to that isolated from pig heart (Sigma). In mutant binary CS–OAA complexes, only Asp375Gly resembles wild-type enzyme. Neither Asp375Glu nor His274Gly generates as large a chemical shift change in the binary complex. His274Gly actually shows two OAA carbonyl resonances with an approximate 2:1 intensity (area) ratio in favor of the form with the larger chemical shift. This finding has been confirmed with a second preparation from this mutant.

In ternary complexes, both Asp375 mutants behave like wild type. The maximum chemical shift increase of ~7 ppm is experienced by the OAA carbonyl in these ternary complexes. However, the major OAA resonance in the ternary complex of His274Gly remains unchanged from its position in the binary complex.

Chemical Shifts of [1-^{13}C]CMCoA Bound to Citrate Synthase Mutants. Binary complex chemical shifts are unaffected

[3] The nonlinear regression of data with two of the three parameters fixed at separately determined values gave a result for K_d = 0.003 ± 0.001 μM. However, inspection of the quality of the data convinced us that this K_d value is only approximate.

Table I: Chemical Shifts and Dissociation Constants for [2-^{13}C]OAA and [1-^{13}C]CMCoA Bound to Citrate Synthase Mutants

	CS–OAA*[a]		CS–OAA*–CMCoA[a]		CS–CMCoA*[a]		CS–OAA–CMCoA*[a]	
enzyme[a]	δ^b	K_d (μM)	δ^b	K_d (μM)	δ^b	K_d (μM)	δ^b	K_d (μM)
WT (Sigma)	204.1[c]	1.2[d]	206.6[c]	–[e]	177.4	51.0[f]	175.2	0.023[f]
WT (clone)	204.6	1.1	206.5	–	177.7	–	175.5	–
Asp375Glu	203.0	9.6	205.8	–	177.7	–	173.1	1.9
Asp375Gly	203.9	3.3	206.1	–	177.9	0.8	171.6	≤0.003
Asp375Asn	–	1.2	–	–	–	–	–	≤0.003
His274Gly	203.0/202.4[g]	5.9	203.1	–	177.5	–	176.4	9.2
free ligand	OAA (carbonyl) 199.8				CMCoA (anion) 178.1		CMCoA (acid) 174.8	

[a] The asterisk indicates which ligand contains the ^{13}C label. [b] Variations in chemical shifts for bound ligands for duplicate determinations are ~0.3 ppm. [c] Kurz et al. (1985). [d] K_d for dissociation of OAA from CS determined by OAA protection against urea denaturation (Zhi et al., 1991). [e] Not determined. [f] K_d for dissociation of CMCoA from CS and CS–OAA determined by CD titration. [g] For CS–OAA two OAA resonances detected, the peak at 203 having about twice the area of that at 202.4.

Table II: Organic Solvent Models for the Effects of Hydrogen Bonding on Carboxyl and Carboxylate ^{13}C Chemical Shifts

solvent	polarity[a] E_T^N	ϵ^a (25 °C)	HBA	HBD	δ^{aq}_{COOH}[c]	$\Delta\delta^{aq}_{COO\text{-}[K+]}$[c]
D_2O	0.991	78.3	+	+	176.4[c]	181.2[c]
TFE	0.898	26.1[b]	+–	++	+1.9	–1.2
MeOD	0.762	52.7	+	+	–1.2	–1.1
MeCN	0.460	35.9	+	–	–4.0	–6.2
acetone	0.355	20.6	+	–	–4.4	–6.4
$CDCl_3$	0.259	4.8	–	+ (weak)	<–3.8	–3.8

[a] Data given are for the ^{1}H solvent (except for that for D_2O) and are taken from Reichardt (1988). E_T^N is an empirical parameter of solvent polarity based on the transition energy at 25 °C of the long-wavelength absorption of a standard pyridinium *N*-phenoxide betaine dye, $E_T(30)$. Water (^1H_2O) is given a value of 1.000 on this scale. [b] Data for TFE are from Mukerjee and Grunwald (1958). [c] The first entries in these columns are the absolute values for the chemical shifts in aqueous solutions. The data for the other solvents are expressed as differences from these values.

by any of the mutations studied—even that of the tight-binding Asp375Gly mutant. Ternary complex chemical shift values of Asp375Glu and Asp375Gly mutants are significantly smaller than those of wild type, while that of His274Gly is significantly larger. Note the very low value of the chemical shift in the Asp375Gly mutant, in which one potential hydrogen bond partner to the carboxyl has been removed, and the intermediate value of the Asp375Glu mutant, in which one potential hydrogen bond might have a different geometry to accommodate the extra methylene group.

Carboxyl–Carboxylate Chemical Shifts in Various Organic Solvents—Models for the Effects of Hydrogen Bonding. It is necessary to develop models for the chemical shift of an enzyme-bound ligand containing a carboxyl or carboxylate when we expect the hydrogen-bonding interactions to be significantly perturbed (Table II). The absolute value of the chemical shift of a particular carboxyl- or carboxylate-containing compound is mainly determined by the covalent molecular structure. However, there are very large environmental effects. For the acid, it is well established (Maciel & Traficante, 1960) that among the most important of these environmental influences are the number and strength of hydrogen bonds. In the wild-type CS active sites, the CMCoA carboxyl is isolated from the bulk solvent and two potential hydrogen-bonding partners are available (Karpusas et al., 1990, 1991). In the mutant enzymes, one of these potential hydrogen bond partners has been positionally modified (Asp375Glu) or entirely removed (Asp375Gly, His274Gly).

The early work on the carboxyl chemical shift was done prior to major advances in NMR instrumentation, and sensitivity limitations previously did not always allow direct determination of the monomer chemical shift. The dimerization of acetic acid in many solvents requires determination of the asymptotic value on the dilution curve. In the present study, sequential additions were made until a constant value was obtained. For the carboxylate anion, no previous study of the chemical shift as a function of solvent is available. For acetone, acetonitrile, and chloroform, potassium acetate is not very soluble, requiring the addition of crown ether to effect solution. Even then, the anion chemical shift is dependent on the concentration of crown ether, reaching an asymptotic value as the crown ether concentration is increased. Only the final values reached are reported in the table.

In addition to the values for carboxyl and carboxylate chemical shifts, values of some physical properties of the studied solvents are included in Table II which might have some relevance to the environment of the active site. Values of dielectric constant, ϵ, and normalized solvent polarity values, E_T^N, are given along with a qualitative indication of the hydrogen bond donating/accepting capabilities of each solvent.

DISCUSSION

Acetyl-CoA enol has been proposed as a chemical intermediate in the citrate synthase reaction (Karpusas et al., 1990, 1991). In producing this intermediate, Asp375 is proposed to act as a base, abstracting a methyl proton from acetyl-CoA, while His274 acts as an acid to donate a proton to the acetyl-CoA carbonyl. This scheme is based on the X-ray structure of the solid-state enzyme complex with OAA and an analog of the proposed intermediate, CMCoA (Karpusas et al., 1990), and the X-ray structures of acetyl-CoA with OAA analogs (Karpusas et al., 1991). The *analog* of an uncharged intermediate should be bound in a neutral form regardless of its charge in solution. We have reported solution-state evidence supporting binding of a neutral CMCoA to CS–OAA in the previous paper. We now focus on the two amino acid residues implicated in the production of the proposed intermediate. We report data suggesting that Asp375 and His274 act as general base and acid, respectively, and we give further evidence that bound CMCoA is a neutral analog of a neutral intermediate.

Charge of CMCoA in Mutant CS and CS–OAA Complexes—Interpretation of the CMCoA ^{13}C Chemical Shifts. The chemical shifts of both carboxylates and carboxylic acids are sensitive to the number, strength, and geometry of the hydrogen bonds they form. This sensitivity was first studied by Maciel and Traficante (1960). Both COOH and COO^- can potentially be involved in two hydrogen bonds, the COOH requiring one hydrogen bond donor and one acceptor and the COO^- requiring two hydrogen bond donors. Hydrogen-

bonding interactions exert an overall deshielding effect (Table II) which is approximately additive and independent of whether the solvent donates or accepts the hydrogen bond. Dielectric constant and E_T^N polarity values are of secondary importance to the number of hydrogen bonds. The hydroxylic solvents, water and MeOH, which can serve as hydrogen bond donors and acceptors (HBD/HBA solvents) maximally deshield both COOH and COO^-. HBA solvents, acetonitrile and acetone (Reichart, 1988), cannot hydrogen bond to the COO^- and consequently do not deshield it, while they partially deshield COOH because they can form one of the two possible hydrogen bonds. The hydrogen bond donor solvent $CHCl_3$ (Lemley, 1976) deshields both COOH and COO^- to an intermediate extent by providing one hydrogen bond to either the acid or anion.

Thus, the model required for the binary enzyme CMCoA complex is that of a hydrogen-bonded anion requiring two hydrogen bond donors in a medium with a lower dielectric constant. Only modest chemical shift changes from water, ~1 ppm, are observed in methanol, a solvent providing such an environment. The model for the ternary complex of the wild-type enzyme is a doubly hydrogen-bonded COOH with one donor and one acceptor, in a low dielectric medium. MeOH will serve here as well. The model for the ternary complex in the Asp275Gly mutant requires a single hydrogen-bonded interaction involving a hydrogen bond acceptor for the COOH. Acetone or MeCN may mimic this active site.

The strength of the hydrogen bond is also important. In TFE, an extremely strong HBD solvent (Mukherjee & Grunwald, 1958; Zana, 1974), the acetic acid carboxyl is deshielded more effectively than in water. We would expect hydrogen bonds weaker than those of water to deshield less effectively. The addition of a methylene group in the Asp375Glu mutant may result in less than optimal hydrogen-bonding geometry and thus in less effective deshielding of the protonated carboxyl of CMCoA.

As suggested by the model solvent observations, there seems little doubt that the extremely low chemical shift observed in the Asp375Gly–OAA–CMCoA complex, shielded by 7.5 ppm compared to the CMCoA anion free in aqueous solution, reflects a protonated carboxyl missing one hydrogen-bonding partner. Strong evidence in favor of this interpretation is provided by the closely similar p*I* patterns for ternary complexes of both wild-type cloned and Asp375Gly enzymes (see discussion below). It is also clear as a consequence of the very large hydrogen-bonding effects that carboxyl–carboxylate chemical shifts must be interpreted cautiously. In the present case it is possible to develop a consistent explanation with aid of the model system data. The weight of the evidence (p*I*, K_d, and NMR data) suggests that the shifts observed for Asp375Gly (171.6), Asp375Glu (173.1), and wild type (175.2) all represent a *protonated* carboxyl of CMCoA.

The interpretation of the value of the chemical shift of the ternary complex of His274Gly is more equivocal. It is reasonable to propose that deletion of the general acid believed to donate a proton neutralizing the intermediate will have a similar result with the analog, and we would expect that CMCoA will be anionic in this complex. The observed chemical shift is consistent with that of a carboxylate anion in a somewhat hydrophobic environment. If an anionic complex were in the closed conformation (which completely isolates the active site from the bulk solution), problems would arise with regard to electrical neutrality in the active site. Evidence suggesting that the conformational equilibrium of this mutant is shifted away from the closed form is discussed below.

Isoelectric Focusing and K_d Values for Complexes. (*a*) *Asp375Gly and Asp375Asn.* CMCoA exists as an anion in solutions of pH 7.5 (pK_a = 3.90). As an analog of a neutral acetyl-CoA intermediate enol, CMCoA should be neutral when bound, and a proton must be taken up by the ternary complex. The requirement for proton uptake makes a substantial endergonic contribution to the overall stability of the complex. Assuming a normal initial pK_a, we would expect Asp375 to be anionic while finite concentrations of cationic His274 should be present. (The pK_a of many protein histidine residues is close to neutrality, so substantial concentrations of both charge forms might be expected.) The proton taken up then is likely shared by Asp375 and the CMCoA carboxyl. However, the Asp375Gly mutant could bind a neutral protonated CMCoA without the unfavorable extraction of a solvent proton if the required proton is provided by the putative general acid residue, His274. This expectation is consistent with the observed very tight binding of CMCoA to the mutants Asp375Gly and Asp375Asn (Table I). Since no proton uptake is required when the enzyme produces the actual intermediate, acetyl-CoA enol, the affinity of CMCoA for the Asp375Gly and Asp375Asn mutants may more closely approximate the affinity of the true intermediate for the native enzyme. It is interesting to note that OAA–CoA ternary complexes of these mutants are also the most thermally stable (Zhi et al., 1991).

This explanation requires that the charge on the CMCoA–OAA complex of enzymes in which Asp375 has been changed to a neutral residue would be the same as that on the wild type. While we cannot measure this directly at pH 7.5, we find that the complexes have the same p*I*. (A gel comparing Asp375Gly with wild type is shown in Figure 1.) The strengths of these arguments are further increased by the results with the Asp375Asn mutant. This mutant also shows very tight CMCoA binding ($K_d \leq 0.003$ μM). Its well-focused ternary complex p*I* pattern is very similar to those of wild-type clone and Asp375Gly.

If this sharp focus of the p*I* pattern of these mutants is the signature of the closed form with its active site inaccessible to solvent, a requirement for active site neutrality is reasonable. The apparent neutrality of these complexes along with their low K_d is most easily understood if His274 initially exists as a cation, ready to donate its proton to the inhibitor carboxyl to form a neutral complex.

A possible complication arises from consideration of the protein structure (in the solid state) in the vicinity of His274 (S. J. Remington, personal communication). His274 lies at the amino terminus of an α-helix and experiences the electrostatic field of the positive end of the α-helic dipole. This may lower the pK_a of His274 sufficiently that the proposed imidazolium cation cannot form. A similar situation exists with His95 in the active site of triosephosphate isomerase (Lodi & Knowles, 1991), an enzyme with many similarities to citrate synthase. His95 also lies at the amino terminus of an α-helix. Lodi and Knowles (1991) observed that His95 remains neutral in the free enzyme and all stable complexes. Since the second pK_a would also be lowered, they proposed that His95 acts as a general acid to donate a proton to form an enediol intermediate transiently generating an imidazolate anion. If this is the case with citrate synthase, Asp375 is still required to act as a base, removing the methyl proton from acetyl-CoA. A neutral intermediate acetyl-CoA enol still would result, but His274 would carry a negative charge. The place of the CMCoA ternary complex in such a scheme is uncertain. Furthermore, the extreme stability of the complexes with the Asp375Gly and Asp375Asn mutants is

hard to explain. The neutral CMCoA complexes whose solid-state structures have been studied (Karpusas et al., 1990, 1991) and which we observe in solution seem to require that His274 initially be cationic. These neutral complexes, however, may be analogs of an intermediate *which is not on the reaction pathway*. In the case of triosephosphate isomerase, theoretical calculations (Bash et al., 1991) predict that a reaction intermediate with His95 positively charged is too stable to be kinetically viable. NMR studies with ^{13}C and/or ^{15}N-labeled histidine, similar to those of Lodi and Knowles (1991), will be required to address this issue for citrate synthase.

Wilde et al. (1990) have proposed a mechanism for citrate synthase in which His274 acts as the base and Asp375 acts as the acid. This scheme seems incompatible with the low pK_a for His274 suggested by the solid-state X-ray structure. In addition, it would require Asp375 to have an abnormally high pK_a (in order to have a proton to provide). Furthermore, this scheme would lead to retention of configuration of the remaining protons at the attacking carbon atom (Karpusas et al., 1990) rather than the experimentally observed inversion of configuration (Eggerer et al., 1960).

The analogous Asp to Gly site-directed mutant of the *E. coli* enzyme, Asp362Gly, has been prepared and studied (Hanford et al., 1988). In marked contrast to our results with the nonallosteric citrate synthase, Hanford et al. (1988) report that the mutant enzyme–OAA complex is unable to bind CMCoA. These authors assessed CMCoA binding by testing for coelution of radiolabeled CMCoA with CS protein on a Sephadex G-75 column equilibrated in 0.7 mM OAA, 20 mM Tris-HCl, and 0.1 mM EDTA, pH 8.0. Under these low ionic strength conditions in the absence of KCl, we have shown (Kurz et al., 1992) that the *E. coli* CS–OAA–CMCoA complex is *not* an analog of a reactive intermediate complex in the same way as the pig heart CS–OAA–CMCoA complex. The chemical shift of bound CMCoA carboxyl in the *E. coli* CS–OAA–CMCoA complex remains close to that of the anion until KCl is added. CMCoA binding to this mutant should be reevaluated in the presence of KCl.

(*b*) *Asp375Glu.* The addition/deletion of a methylene group in a catalytic residue would seem to be a relatively benign change. Such is the case with dihydrofolate reductases where the active site acidic residue occurs as either Asp or Glu, depending upon the organism (Groom et al., 1991; Birdsall et al., 1989). However, for other enzymes the presence or absence of a methylene group is surprisingly important. For triosephosphate isomerase, its deletion (Glu165Asp) results in a 1000-fold reduction in specific activity (Raines et al., 1986). For citrate synthase, an additional methylene group (Asp375Glu) reduces the specific activity by over 400-fold (Alter et al., 1990). While the effect of the mutation in the case of citrate synthase primarily resides in k_{cat}, it has not yet been shown whether it causes reductions in the stabilities of intermediates, transition states, or both. Such studies are in progress for the pig heart enzyme.

The broad p*I* for *all* complexes of the Asp375Glu enzyme may give insight into the catalytic lesion induced by this mutation. We have speculated (Kurz et al., 1992) that the broad and narrow p*I* forms observed with the wild-type enzyme and its complexes are the respective signatures of the "open" and "closed" forms observed in the solid-state, crystallographically. The absence of well-focused p*I* patterns for the Asp375Glu mutant ternary complex suggests that this enzyme is impaired in its ability to access the closed conformation even in the presence of an analog of a reactive intermediate.

The catalytic cycle of citrate synthase is composed of two reactions: formation of a C–C bond followed by hydrolysis of a C–S bond, both occurring in a single active site catalyzed by the same residues. The strategies required to optimize each reaction rate might reasonably be expected to differ. It is tempting to speculate that the two conformational states observed for the enzyme are related to this catalytic switch. The function of the closed conformation may be the proper positioning of catalytic residues and/or it may be that exclusion of solvent water from the active site is required during the ligase reaction to avoid futile acetyl-CoA hydrolysis. The function of the open form may be merely to allow the release of products, or it may be required to admit solvent water for the hydrolysis of the intermediate citryl-CoA. In either case, the ability of the enzyme to reach the closed conformation at the proper time is essential to efficient turnover. Apparently, an extra methylene group in the Asp375Glu mutant, a modest local structural change, seriously interferes with a necessary global conformational change.

(*c*) *His274Gly.* This mutant also shows broad p*I* bands for both the free enzyme and its complexes. The same arguments used with the Asp375Glu mutant may apply, and this enzyme is also impaired in its ability to reach the closed conformation. In this case, however, the reasons may be electrostatic rather than steric. To remain electrically neutral, a closed conformation for the ternary complex would have to include two protons (one for Asp375 and one for CMCoA) or two counterions would have to be bound along with the anions. This does not appear to be possible, and this enzyme may remain open.

Ability of Mutant CS to Polarize OAA—Interpretation of the [2-^{13}C]OAA Chemical Shift. The catalytic strategy of citrate synthase involves the activation of *both* substrates. Polarization of the C=O bond of OAA with substantial positive charge development at the carbonyl carbon has been demonstrated by ^{13}C NMR and FTIR spectroscopy (Kurz et al., 1985; Kurz & Drysdale, 1987). This polarization, which is evident even in the binary CS–OAA complex, reaches its greatest extent in the ternary complex with CMCoA. The ~7 ppm increase in the ^{13}C shift of the OAA carbonyl in the ternary CS–OAA–CMCoA complex may represent about a 30% decrease in the OAA C=O π-bond order.

(*a*) *Asp Mutants.* In the binary CS–OAA complex, the Asp375Glu mutant achieves less OAA polarization than the wild-type enzyme while the Asp375Gly mutant is not significantly different from wild type. In ternary complexes, both Asp mutants are able to achieve full polarization of the OAA carbonyl. These results may be explained in terms of the relative difficulty experienced by the Glu mutant in achieving the closed conformation. The energy released by OAA binding may be insufficient to position the residues involved in substrate destabilization, an endergonic interaction. However, the much greater energy release accompanying transition-state analog binding is sufficient to drive that interaction.

(*b*) *His274Gly.* In the binary complex, OAA has two well-defined resonance positions, implying two binding modes. The intensities (areas) of the two resonances are approximately in a 2:1 ratio, with the resonance at higher chemical shift having the greater area. In the ternary complex, only one resonance is evident, unshifted from the major resonance of the binary complex. In the wild-type enzyme active site, His274 is in contact with the OAA methylene (Karpusas et al., 1990). The smaller Gly residue may remove a steric constraint, not only allowing binding in more than one mode but allowing the

substrate to escape from the fundamentally destabilizing interactions which lead to substrate activation through polarization of the C=O bond. In addition, His274 is near the "hinge" region of the CS molecule. We propose that His274Gly is impaired in its ability to achieve this conformation change (supported by lack of focus of any complex on IEF gels). Since His274 is also a catalytic residue, it is not possible to separate the deleterious consequences of interference in the hinge motion from those resulting from deletion of a general acid stabilizing the intermediate. Mutants designed to interfere with the formation of the hinge motion will be prepared and characterized.

Conclusion. Structural and solution studies indicate that the activated form of acetyl-CoA in the citrate synthase reaction is a neutral enol. The catalytic residues Asp375 and His274 act, respectively, as general base and general acid to stabilize this intermediate. The presence of His274 is necessary for the enzyme to polarize the carbonyl of OAA, and His274 plays a role in the large conformation change which accompanies the catalytic cycle.

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Registry No. OAA, 328-42-7; CS, 9027-96-7; His, 71-00-1; Asp, 56-84-8; acetyl CoA, 72-89-9.