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Proposed Mechanism for the Condensation Reaction of Citrate Synthase: 1.9-Å Structure of the Ternary Complex with Oxaloacetate and Carboxymethyl Coenzyme A†

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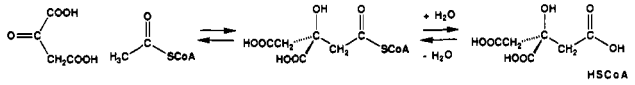
Received November 21, 1989; Revised Manuscript Received January 11, 1990

ABSTRACT: The crystal structure of the ternary complex citrate synthase–oxaloacetate–carboxymethyl coenzyme A has been solved to a resolution of 1.9 Å and refined to a conventional crystallographic *R* factor of 0.185. The structure resembles a proposed transition state of the condensation reaction and suggests that the condensation reaction proceeds through a neutral enol rather than an enolate intermediate. A mechanism for the condensation reaction is proposed which involves the participation of three key catalytic groups (Asp 375, His 274, and His 320) in two distinct steps. The proposed mechanism invokes concerted general acid–base catalysis twice to explain both the energetics of the reaction and the experimentally observed inversion of stereochemistry at the attacking carbon atom.

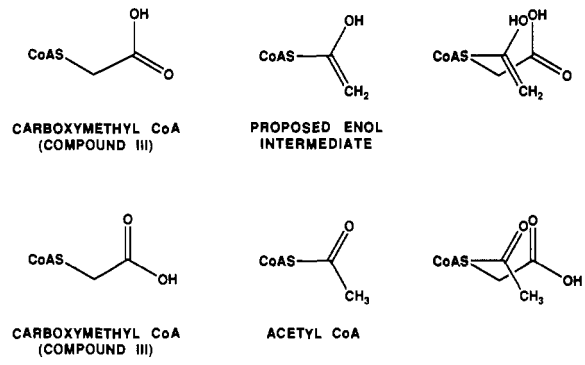
Citrate synthase (CS)[1] is a member of a small class of enzymes that can directly form a carbon–carbon bond in the absence of metal ion cofactors. The enzyme catalyzes the Claisen condensation of acetyl coenzyme A and oxaloacetate to form citrate and coenzyme A as the first step in the citric acid cycle [for reviews, see Srere (1972), Weitzman and Danson (1976), and Wiegand and Remington (1986)]. A great deal of experimental data on the nature of this reaction has been gathered. The reaction is widely believed to proceed through a noncovalently bound intermediate, citryl-CoA, and was thus thought to be a two-step reaction (condensation followed by hydrolysis):



OXALOACETIC ACID ACETYL CoA CITRYL CoA CITRIC ACID

Eggerer and colleagues (Eggerer et al., 1970) have demonstrated that the reaction proceeds with inversion of configuration on the methyl group of acetyl-CoA and have provided evidence that the enolization of acetyl-CoA is the rate-limiting step of the reaction (Eggerer, 1965). They have also shown that citryl-CoA can be observed to undergo either the forward or reverse half-reaction (Eggerer & Remberger, 1963). Finally, these workers have studied a number of inhibitors of the synthase reaction and concluded that compound III (carboxymethyl-CoA) is a transition-state analogue. It is a potent inhibitor of the reaction (K_i = 0.08 μM; Bayer et al., 1981) and was proposed by these authors to resemble the enol of acetyl-CoA as diagrammed in Chart I (top line); i.e., the tetrahedral configuration of the "extra" methylene group still allows the enzyme to make essentially the same interactions with both compounds. An alternative correspondence, in which the inhibitor resembles the substrate acetyl-CoA, is shown in the second line of Chart I.

Chart I



The structure of pig heart and chicken heart citrate synthase has been determined by X-ray crystallography for three different crystal forms and a variety of binary and ternary complexes (Remington et al., 1982; Wiegand et al., 1984; Wiegand & Remington, 1986). These studies have led to a tentative identification of catalytically important amino acid side chains (His 274, His 320, and Asp 275) and have also revealed the enzyme to be capable of assuming three different conformations, one "open" and two "closed" conformations. The studies suggested that part of the catalytic efficiency of the enzyme results from electrostatic destabilization (polarization) of the substrate oxaloacetate. Handford et al. (1988) have clearly demonstrated by site-directed mutagenesis and kinetic analyses that Asp 362 of *Escherichia coli* CS (believed to correspond to Asp 375 of pig heart CS) is essential for activity and for the binding of carboxymethyl-CoA but not oxaloacetate.

†This research was supported in part by grants from the National Science Foundation (DMB-8817438) to S.J.R. and (DMB-8717730) to B.B. and a grant to the Institute of Molecular Biology from the Lucille P. Markey Charitable Trust.

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[1] Abbreviations: CoA, coenzyme A; CS, citrate synthase; citryl thioether CoA, (3*S*)-3,4-dicarboxy-3-hydroxybutyl coenzyme A; CMCoA, carboxymethyl coenzyme A; rms, root mean square.

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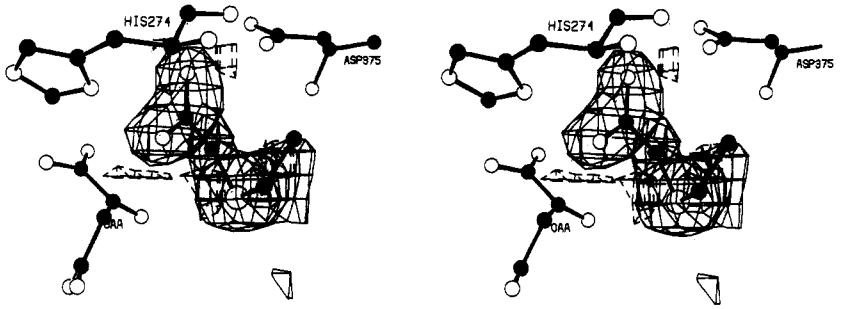

FIGURE 1: Stereoview of the original $F_o - F_c$ difference electron density map contoured at one standard deviation. The atoms of bound oxaloacetate were included in the calculation of phases. Also shown are the side chains of His 274 and Asp 375 and the atoms of carboxymethyl coenzyme A, which were not used to calculate phases. Carbon atoms are filled, oxygen, nitrogen, and sulfur are open, and the sulfur is drawn with a radius twice that of the lighter atoms.

Hanson and Rose (1975) summarized a great deal of experimental data on the stereochemical course of a variety enzymatic reactions, including the Claisen condensations. They pointed out that there are seven enzymes that catalyze reactions similar to that of citrate synthase for which detailed sterochemical information is available. In each case the reaction proceeds with inversion of configuration of the protons on the attacking carbon atom.

Here, we discuss a high-resolution structure of the ternary complex citrate synthase–oxaloacetate–carboxymethyl-CoA. This structure and the results of our previous studies suggest that a single conformation of the enzyme is capable of catalyzing three distinct and very different steps of the overall reaction (enolization, condensation, and hydrolysis).

In order to establish a working hypothesis, a detailed mechanism is proposed for the first two steps of the reaction. The proposal suggests an explanation for the energetics and the stereochemical course of the reaction. We further propose that the basic features of this mechanism will be valid for other enzymes that catalyze this class of Claisen condensations.

Methods and Results

The ternary complex chicken heart (Sigma) citrate synthase–oxaloacetate–carboxymethyl-CoA crystallizes isomorphously to and under the same conditions as the product complex containing citrate and CoA previously solved and refined to 1.7-Å resolution (NPH6 model; Remington et al., 1982; Table I). The enzyme (8 mg/mL in 0.7 M citrate, pH 6) was preincubated with 2.5 mM oxaloacetate and 2.5 mM carboxymethyl-CoA for 1 h before crystallization by vapor diffusion against 1.1 M sodium citrate, pH 6. Data were collected to 1.9 Å by oscillation photography (Schmidt et al., 1981; Table I) on a conventional rotating-anode X-ray generator. An $F_o - F_c$ difference Fourier electron density map was calculated by use of phases derived from the product complex CS–citrate–CoA with atoms removed from citrate and CoA that were not expected to have counterparts in the complex. The largest peak in the map clearly revealed the location of the sulfur atom and the bifurcation of the electron density at the carboxylate of CMCoA (Figure 1). A model, constructed with the FRODO programs (Jones, 1978), was refined to a conventional crystallographic R factor of 0.185 (CMCA model, Table I) with the TNT program package (Tronrud et al., 1987). For subsequent structural comparisons, the structures of the product complex CS–citrate–CoA and an analogue of the reaction intermediate complex CS–citryl thioether CoA (Remington et al., 1982) were re-refined against the original data sets with the TNT programs (Table I).

The new structure surprised us. Most surprising was that the structure of the enzyme is to the limits of accuracy of this investigation virtually identical in the three crystal structures

Table I: Experimental Data[a] and Atomic Models

Chicken Heart Citrate Synthase–Oxalaocetate–Carboxymethyl Coenzyme A Complex (CMCA)			
space group	$C2$	unit cell	
no. of crystals	2	a (Å)	104.0
total reflections	61 965	b (Å)	78.1
no. of film packs	45	c (Å)	58.3
unique reflections	30 344	β (deg)	78.9
		R_{merge}[b]	8.6

Crystallographic Models

model	R factor	resolution (Å)	deviation from ideality (rms) bonds (Å)	angles (deg)	planarity (Å)	bad contacts (Å)
CMCA	0.185	1.9	0.011	2.5	0.026	0.047
CIC6	0.110	2.5	0.008	3.2	0.022	0.056
NPH6	0.190	1.7	0.010	2.2	0.033	0.103

rms Coordinate Differences between All Protein Atoms (Å)

NPH6–CIC6	0.18	CIC6–CMCA	0.14
NPH6–CMCA	0.13		

[a] Experimental data for models CIC6 (CS–citryl thioether CoA) and NPH6 (CS–citrate–CoA) are described in Remington et al. (1982). [b] R_{merge} gives the average disagreement in percent of multiple measurements of the same intensities.

(Table I). This was unexpected, as the structures should represent three different states of the enzyme, namely, the state preceding condensation (with bound oxaloacetate and carboxymethyl-CoA), the state containing bound intermediate (with bound citryl thioether CoA), and the product complex (with bound citrate and CoA).

As shown in Figure 2, the conformation of oxaloacetate is clearly revealed in the final $2F_o - F_c$ electron density map, confirming previous suggestions from a 2.9 Å resolution study (Wiegand et al., 1984) that the molecule binds to the enzyme analogously to citrate. The molecule makes salt bridges or hydrogen bonds to three arginine (239, 401, 421) and two histidine (238, 320) residues (Figure 2b). The proposed mechanism requires that His 320 be protonated for the reaction to occur, so that the oxaloacetate molecule is surrounded by at least four positively charged electron-withdrawing groups. Thus, the environment at the carbonyl carbon is extremely polar. It is also clear from the observed geometry that oxaloacetate is bound as the keto rather the enol tautomer, confirming the results of Englard (1958).

The electron density map revealed that the carboxylate of CMCoA is hydrogen bonded to two residues, Asp 375 and His 274 (Figure 3) with nearly perfect hydrogen-bond geometry (Figure 3b), with an important exception. This is at the carboxylate O92 of the inhibitor. As is indicated in Figure 3a, the O92–Asp 375 OD1 hydrogen bond is far out of the plane of the O92 carboxylate (an unfavorable situation) and

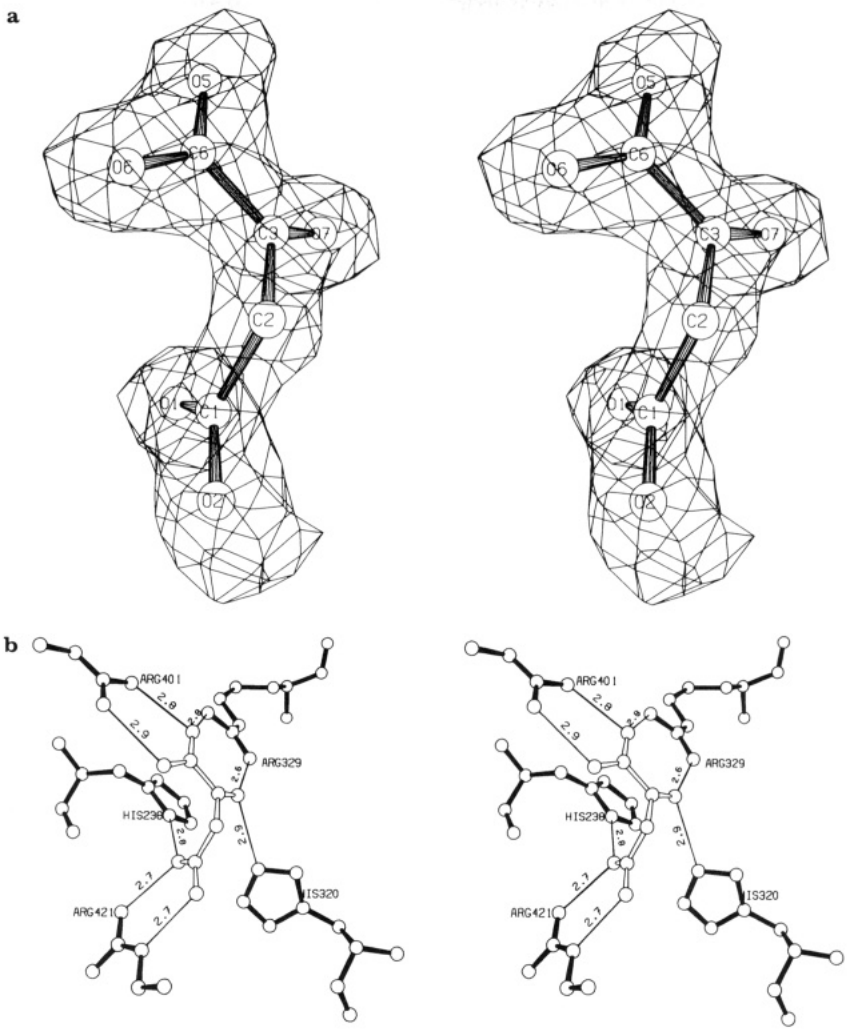

FIGURE 2: (a) Final $2F_o - F_c$ electron density map contoured at one standard deviation, showing atom labels and conformation of bound oxaloacetate as the keto rather than the enol tautomer. (b) Conformation of bound oxaloacetate and neighboring residues. The thin bonds represent hydrogen bonds or salt bridges with the indicated lengths in angstroms. As discussed in the text, the molecule is surrounded by positively charged electron-withdrawing groups, which very likely induce a large positive partial charge at C3.

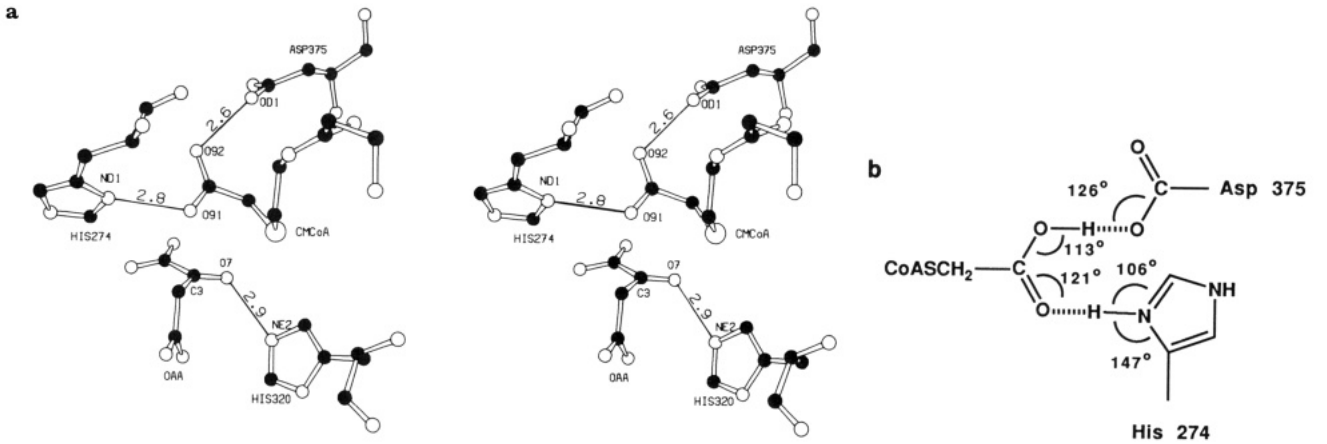

FIGURE 3: (a) Enzyme-bound conformation of the carboxymethyl "tail" of carboxymethyl coenzyme A showing hydrogen bonds or salt bridges to Asp 375 and His 274. The hydrogen bond from His 320 to the carbonyl oxygen (O7) of oxaloacetate is also indicated. The largest atom is sulfur. (b) Hydrogen bond angles in (a). Note that the hydrogen bonds are not coplanar as implied by the figure. The $CoASCH_2$–C–O–H dihedral angle to Asp 375 is close to 90°.

resembles more closely the configuration that might obtain in the transition state if O92 were a methyl group.

The carboxymethyl moiety is in van der Waals contact with the oxaloacetate molecule, and the plane of this moiety is about 60° from parallel to the plane of the carbonyl carbon of oxaloacetate. To the extent that this structure might represent the transition state of the enolization reaction, it clearly indicates that two groups (the histidine and the aspartate) are intimately and simultaneously involved. Both substrates and the reactive site groups are completely inaccessible to solvent.

Atomic coordinates resulting from this study have been deposited in the Brookhaven Data Bank (Bernstein et al.,

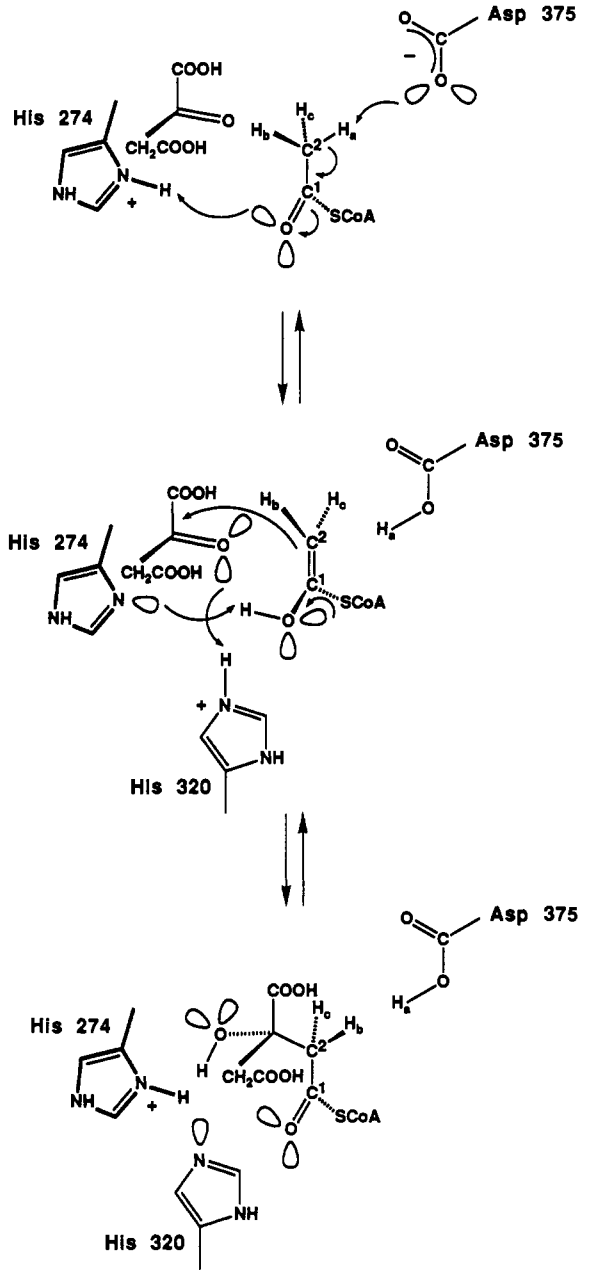

FIGURE 4: Schematic of the proposed reaction sequence of the condensation reaction of acetyl coenzyme A and oxaloacetate to form the intermediate citryl coenzyme A. The reaction is proposed to proceed via simultaneous general acid–base catalysis (top) to form a neutral enol intermediate (center) which then attacks the carbonyl carbon of oxaloacetate (bottom). The experimentally observed inversion of configuration of the protons on the methyl group of acetyl-CoA is indicated by the labeling of the hydrogen atoms. Solely for the purposes of discussion (see text), the two carbons of the enol are labeled 1 and 2 as shown.

1977). For structural studies at this resolution, the rms coordinate error is estimated to be about 0.2 Å.

Proposed Mechanism for the Condensation Reaction

(*Step 1*) *Enolization.* The arrangement of the carboxymethyl moiety in the active site of citrate synthase suggests a possible mechanism for the first step of the synthase reaction (shown schematically in Figure 4) whereby a proton is abstracted from the methyl group of acetyl-CoA via simultaneous general acid–base catalysis, resulting in a neutral enol intermediate. Either His 274 or Asp 375 could act to protonate the carbonyl oxygen of the acetyl group, while the other residue simultaneously accepts a proton from the methyl group. The enzyme thus can form the neutral enol without generating a high-energy enolate or protonated ketone intermediate, as has been proposed by several authors [e.g., Lienhard and Jencks (1965), Spector (1972), and Loehlein-Wehrhahn et al. (1983)].

However, in order to establish whether this is a possible mechanism, three questions must first be answered: (1) Is it possible to demonstrate that the proposed transition state of acetyl-CoA can be accommodated in a manner analogous to the arrangement shown in Figure 3? (2) If so, which of the two oxygens (O91 and O92 in Figure 3a) of the carboxylate of carboxymethyl-CoA corresponds to the methyl group of the acetyl-CoA? (3) What are the individual roles of the aspartyl and the histidyl side chains?

To address the first question, a hypothetical model of the proposed transition state was constructed, and the geometry was idealized with the TNT package. In constructing this model, it was assumed (for reasons discussed below) that the histidyl side chain is the acid which protonates the carbonyl oxygen of acetyl-CoA. A superposition of the most pertinent atoms of the hypothetical and observed complexes is shown in Figure 5. It appears that the freely rotatable single bonds allow the "extra" methylene group of CMCoA to be accommodated, permitting the same enzyme–substrate interactions to occur with acetyl-CoA as with the inhibitor.

The second and third questions can be addressed with experimental evidence. If one were to assume that the histidine is the base that abstracts the proton from the methyl group, then as seen in Figure 3, one would predict that the stereochemistry of the remaining protons on the attacking carbon would be preserved in contradiction to experimental evidence (Eggerer et al., 1970). This follows as a result of the angle between His 274 ND1 (the site of proton abstraction), the attacking carbon (which would correspond to O91), and the carbonyl carbon of oxaloacetate, which is 93°. However, if it is the aspartate that acts as the base, the proton would be removed from the side of attacking carbon directly opposite the oxaloacetate molecule (Figure 3), forming a planar intermediate as diagrammed in Figure 4. Subsequent attack (in step 2) by the 2-carbon of the enol upon the carbonyl carbon of oxaloacetate would require a rotation of the enol moiety of approximately 90° about the His 274 ND1–O hydrogen bond, and a movement of about 3 Å of the attacking carbon, resulting in the intermediate citryl-CoA. The stereochemistry of the latter scenario leads directly to the inversion of configuration of the methyl protons.

Step 1 requires the histidine to be protonated and positively charged and the aspartate to be unprotonated and negatively charged. The pK_a's of the histidine and the aspartate, if unperturbed by the active site environment, naturally favor this equilibrium situation by more than 100:1 at neutral pH.

(*Step 2*) *Condensation.* The condensation reaction is more speculative; nevertheless, several lines of evidence are in support of this step as diagrammed in Figure 4. The first is that the reaction requires the carbonyl oxygen of oxaloacetate to become protonated, and His 320 (Figure 3) is obviously in an ideal position to accomplish this task. Second, the intermediate analogue complex (CS–citryl thioether CoA, Figure 6a) shows the sulfur to be distal to the histidine, while the product complex (CS–citrate–CoA) clearly shows His 274 to be hydrogen bonded to O3 of citrate (Figure 6b), which must be deprotonated in citryl-CoA. This suggests that the attack of 2-carbon of the enol upon the carbonyl carbon of oxaloacetate is guided by the hydrogen bond to His 274 ND1 (i.e., a rotation about this bond), which would then accept the proton

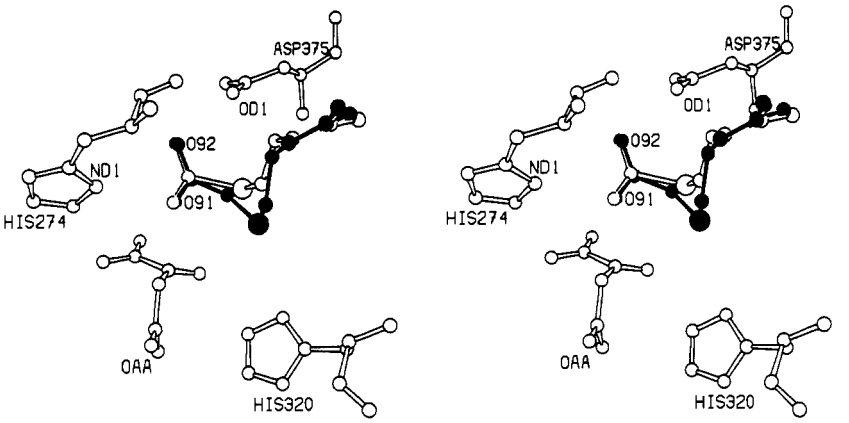

FIGURE 5: Hypothetical model of the enolization transition state of acetyl coenzyme A (open bonds and atoms) superimposed on the observed conformation of carboxymethyl coenzyme A (filled bonds and atoms) together with catalytic groups on the enzyme. The sulfur atoms are drawn with larger radii for clarity. It is proposed that the atom labeled O92 in carboxymethyl-CoA corresponds to the methyl group of acetyl-CoA. OAA is oxaloacetate.

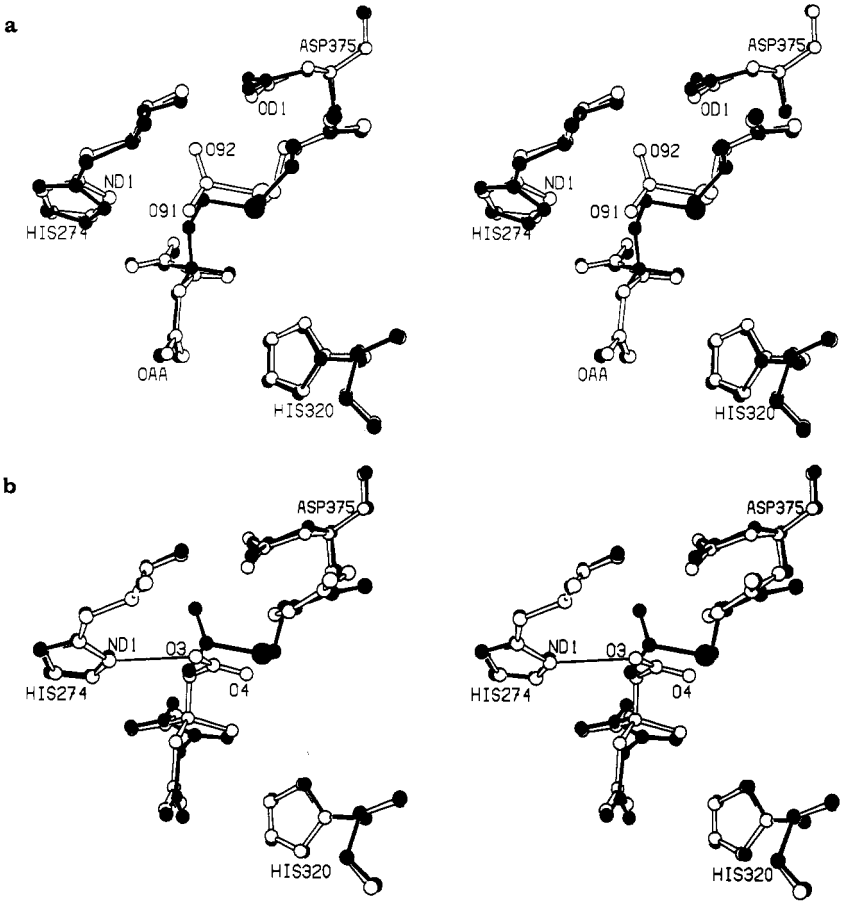

FIGURE 6: (a) Hypothetical model of the enolization transition state of acetyl-CoA (open bonds and atoms) as in Figure 5, superimposed on the observed structure of the analogue of the reaction intermediate citryl thioether coenzyme A (CIC6, filled bonds and atoms). It is important to note that the sulfur (largest atom) is distal to His 274. The structures represent the proposed states of the enzyme immediately before and after the condensation reaction. Catalytic groups on the enzyme are in observed conformations in both cases. (b) Hypothetical model of the enolization transition state (filled bonds and atoms) superimposed on the observed structure of the product complex containing bound citrate and coenzyme A (open bonds and atoms). As described in Remington et al. (1982) the two terminal atoms of coenzyme A (including the sulfur) are not visible in the electron density map and are disordered. The thin bond represents a hydrogen bond between His 274 and O3 of citrate.

from the enolic oxygen as required by the overall reaction. Inspection of the model reveals that there is sufficient room in the active site for the proposed rotation to occur.

We cannot exclude the possibility that the roles of His 274 and Asp 375 are the reverse of those proposed in step 1. However if so, straightforward proton recycling in step 2 could not occur, and the course of the reaction is much more awkward. Also as stated, to account for inversion of stereochemistry at the attacking carbon, one would be forced to postulate a rather different binding configuration for acetyl-CoA than that seen in the present complex.

Both of these steps invoke general acid–base catalysis to avoid generation of charged intermediates. In solution, such a mechanism is a fifth-order reaction involving two acids, a base, and the two reacting species. Thus, it seems possible that a great deal of the rate enhancement due to citrate syn-

thase is entropic, essentially the reduction of this fifth-order reaction to unimolecular on the enzyme surface.

Discussion

Jencks has delineated several possible mechanisms for acid–base catalysis of intermolecular reactions. The following summary is adapted from several papers by Jencks (1981, 1980, 1976).

(*1*) *Sequential Stepwise Mechanism.* A very stable intermediate substrate cation or anion can easily be formed, freely diffuse, and subsequently be converted to a neutral intermediate by encounter with a base or an acid.

(*2*) *Trapping Mechanism.* A less stable shorter-lived intermediate substrate cation or anion is rapidly trapped to form a neutral intermediate by encounter with a base or an acid.

(*3*) *Preassociation Mechanism.* The intermediate substrate cation or anion is so unstable that it cannot freely diffuse before it decomposes back to starting materials. Therefore, the substrate + acid catalyst + base catalyst must all be present in the solvent cage in order for a productive reaction to occur. The reaction is nevertheless stepwise and proceeds through a cation or anion intermediate.

(*4*) *Concerted Mechanism.* The intermediate substrate cation or anion is so unstable that its formation is incompatible with the observed reaction kinetics. To avoid the formation of the intermediate, the reaction proceeds by concerted general acid–base catalysis.

Intermolecular acid–base catalysis of the enolization of acetone has been shown to have a significant component of the trimolecular concerted reaction (Hegarty & Jencks, 1975). For example, the rate law for enolization of acetone catalyzed by acetic acid and acetate in water has a second-order acid-catalyzed term with rate constant $k_A = 4.8 \times 10^{-6}$ M^{-1} min^{-1}, a second-order base-catalyzed term with rate constant $k_B = 12.5 \times 10^{-6}$ M^{-1} min^{-1}, and a third-order acid–base-catalyzed term with rate constant $k_{AB} = 12.6 \times 10^{-6}$ M min^{-1}. It was suggested, on the basis of much solid evidence, that the trimolecular reaction is probably a truly concerted process in which acid catalyst + base catalyst + acetone all participate in a single concerted step to form the enol without passing through any intermediate enolate or protonated ketone.

If concerted general acid–base catalysis is so important in the enolization of acetone with $pK_a = 20$ (House, 1972) in an intermolecular trimolecular reaction, then it is certainly reasonable to consider such a pathway in the pseudo-unimolecular enzyme-catalyzed enolization of acetyl-CoA, which is estimated to have a pK_a comparable to that of acetone [e.g., Lau et al. (1988)]. The basic argument is that the enolate or protonated ketone of acetyl-CoA is such a high-energy intermediate that it is incompatible with the observed kinetics. Similar arguments can be made for the subsequent steps in the Claisen condensation catalyzed by citrate synthase, and these form the basis for the proposed mechanism.

A deeper analysis of the proposed mechanism reveals the arrangement of functional groups and substrates in the active site of citrate synthase to be beautifully in accord with commonly accepted, but seldom demonstrated, ideas about the roles of stereoelectronic control and acid–base catalysis in enzymic catalysis. The spatial arrangements of the base (aspartate 375), the acid (histidine 274), and the substrate (acetyl-CoA) provide nearly optimal orbital overlap for proton transfers in the transition state of the enolization reaction as follows.

An in-line proton transfer between the carboxylate of Asp 375 and the methyl group of acetyl-CoA would transfer the proton to a lone pair on the carboxylate which is orthogonal to the π electron system of the carbonyl of the carboxyl group of Asp 375; i.e., the lone pair is not conjugated with the π system of the carbonyl of Asp 375. The advantage of this is that the proton transfer can proceed without disrupting the π conjugation of the carboxylate of Asp 375.

Consider the C–H bond that is broken α to the carbonyl of acetyl-CoA. An in-line proton transfer between the carboxylate of Asp 375 and the CH_3 of acetyl-CoA would allow the electron pair that remains on the acetyl group to directly become fully conjugated to the carbonyl of acetyl-CoA; i.e., the C–H bond that is being broken is aligned for nearly optimal orbital overlap with the π system of the carbonyl of acetyl-CoA. This is the lowest energy pathway because the developing negative charge on the α-carbon of acetyl-CoA is stabilized by conjugative delocalization of that charge to the electronegative oxygen of the carbonyl of acetyl-CoA.

An in-line proton transfer between the imidazole N–H of His 274 and the carbonyl oxygen of acetyl-CoA would transfer the proton to a lone pair on the carbonyl oxygen which is orthogonal to the π electron system of the carbonyl of acetyl-CoA. The advantage of this is that the proton transfer can proceed without disrupting the π conjugation of the carbonyl with the electron pair that is produced by deprotonation of the α-carbon of acetyl-CoA. On the basis of the observed stereochemistry of the hydrogen bonds between His 320 and the carbonyl oxygen of oxaloacetate and His 274 and citrate, the same arguments for in-line proton transfer and stereoelectronic control can be made for the transition state of the actual condensation step described as step 2.

It is interesting to examine the proposed mechanism in terms of the general principles proposed by Hanson and Rose (1975) for the efficiency of enzymatic catalysis based on principles of natural selection. These are as follows: (1) minimal number rule, the enzyme has a minimum number of catalytic groups, leading to one site that can catalyze multiple reaction steps and permit proton recycling; (2) maximal separation rule, pairs of catalytic groups that operate in concert are located on opposite sides of the enzymatic cleft; (3) minimal motion rule, minimum motion of catalytic groups and/or intermediates takes place during the enzymatic reaction.

If the proposed mechanism is correct, citrate synthase is a spectacular example of these principles. The reaction requires just three side chains (two histidines and an aspartate) which act in pairs during two very different steps of the reaction. In each step, the two catalytic groups are located on opposite sides of the enzymatic cleft, and only proton motion is required. In addition, the proton donated by His 274 is accepted again in the second step of the reaction (Figure 4) and is hence recycled. Finally, the only motion of atoms other than protons is the movement of a single carbon atom of about 3 Å.

An unresolved question concerns the role of the very large conformational changes that take place in citrate synthase. The structures of one open and two different closed forms of the enzyme (the open and closed forms differ in the relative arrangement of a large and a small domain through, roughly speaking, an 18° rotation) have been reported (Remington et al., 1982; Wiegand et al., 1984). Due to the total inaccessibility to solvent of substrates and reactive site groups in the closed form, Remington et al. (1982) hypothesized that the open form was a substrate entry/product release form, which still seems to be a viable proposal. However, Wiegand and Remington (1986) suggested that the differences between the two closed forms might reflect states of the enzyme with different catalytic abilities (e.g., hydrolysis versus condensation). This suggestion is inconsistent with the results discussed

here. Further crystallographic and solution studies will be necessary to resolve this issue.

The proposed mechanism predicts that the condensation reaction proceeds in two distinct steps, and these should in principle be separable. It also predicts that the reaction proceeds through a neutral intermediate. The gene for pig heart citrate synthase has been expressed in *E. coli* (Evans et al., 1988). Through the use of site-directed mutagenesis and other means, experiments are underway to test these hypotheses.

Added in Proof

The structure of the ternary complex of citrate synthase with L-malate and acetyl-CoA has been determined to 2.2-Å resolution and is being refined. Acetyl-CoA binds to the enzyme essentially as predicted, with the presumed carbonyl oxygen of the acetyl group hydrogen bonding to His 274. The presumed methyl group makes van der Waals contact with Asp 375. This supports the hypothesis that His 274 acts as an acid in the enolization step, while Asp 375 acts as the base.

Acknowledgments

We thank Drs. Linda Kurz and George Drysdale for a generous gift of the carboxymethyl coenzyme A used in this investigation and Der-Ing Liao for technical assistance.

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