1.9-Å Structures of Ternary Complexes of Citrate Synthase with D- and L-Malate: Mechanistic Implications[†,‡]

Mihail Karpusas, Debra Holland, and S. James Remington*

Institute of Molecular Biology and Departments of Physics and Chemistry, University of Oregon, Eugene, Oregon 97403

Received October 12, 1990; Revised Manuscript Received February 22, 1991

ABSTRACT: The structures of four isomorphous crystals of ternary complexes of chicken heart citrate synthase with D- or L-malate and acetyl coenzyme A or carboxymethyl coenzyme A have been determined by X-ray crystallography and fully refined at 1.9-Å resolution. The structures show that both L-malate and D-malate bind in a very similar way in the presence of acetylCoA and that the enzyme conformation is "closed". Hydrogen bond geometry is suggested to account for the difference in binding constants of the two stereoisomers. The structures suggest that steric hindrance can account for the observation that proton exchange of acetyl coenzyme A with solvent is catalyzed by citrate synthase in the presence of L-malate but not D-malate. The ternary complexes with malate reveal the mode of binding of the substrate acetylCoA in the ground state. The carbonyl oxygen of the acetyl group is hydrogen bonded to a water molecule and to histidine 274, allowing unambiguous identification of the orientation of this group. The structures support the hypothesis that carboxymethyl coenzyme A is a transition-state analogue for the enolization step of the reaction (Bayer et al., 1981) and additionally support proposed mechanisms for the condensation reaction (Karpusas et al., 1990; Alter et al., 1990).

Citrate synthase catalyzes the reversible condensation of an acetyl moiety with oxaloacetate to regenerate citrate in the citric acid cycle. A wide variety of biochemical, physical-chemical, crystallographic, and other studies have extensively characterized the enzyme from a variety of sources [for reviews see Srere (1972), Weitzman and Danson (1976), Wiegand and Remington (1986), and Beeckmans (1984)].

Four different crystal forms of pig heart and chicken heart citrate synthase and a number of binary and ternary complexes have been studied at medium to high resolution [Remington et al., 1982; Wiegand et al., 1984; Karpusas et al., 1990; Liao et al., 1991 (following paper in this issue)]. These studies together with spectroscopic studies of the protein in solution (Srere, 1966; Weidman et al., 1973; Bayer et al., 1981; Kollmann-Koch & Eggerer, 1989) have shown that the enzyme is capable of assuming at least three different stable conformations. One is "open" while the other two are "closed" (Remington et al., 1982; Wiegand et al., 1984; Wiegand & Remington, 1986). The closed forms differ primarily in the arrangement of internal side chains.

In solution, binding of substrates, substrate analogues, or products appears to induce "closed" forms of the enzyme (Srere, 1966; Kollmann-Koch & Eggerer, 1989), but crystal growth studies have revealed an equilibrium between "open" and "closed" forms of the enzyme in the presence of products citrate and coenzyme A or malate and acetylCoA[1] [Remington et al., 1982; Liao et al., 1991 (see discussion in following paper)].

Detailed mechanisms have been proposed for the reaction catalyzed by citrate synthase (Karpusas et al., 1990; Alter et al., 1990). It has been suggested that the "closed" form of the enzyme carries out all catalytic steps while the "open" form is the product release/substrate entry form (Karpusas et al., 1990). Both mechanistic proposals implicate three key catalytic residues (His 274, Asp 375, and His 320) for the condensation reaction, which is thought to proceed through distinct steps. However, these proposals were weakened by the lack of crystallographic data concerning the conformation of bound substrate acetylCoA.

The rate-limiting step of the overall reaction (Eggerer, 1965) is enolization of the acetyl group. This is proposed to take place by coordinated acid/base catalysis in which His 274 protonates the carbonyl oxygen while Asp 375 deprotonates the methyl group to form a neutral intermediate. The protons of the methyl group of acetylCoA are nonexchangeable in aqueous medium, even in the presence of citrate synthase. However, the enzyme-catalyzed incorporation of tritium label into acetylCoA when L-malate (the naturally occuring stereoisomer), but not D-malate, is present has been observed (Eggerer, 1965). This is puzzling because citrate, the normal reaction product, has the D-malate substructure. D-Malate binds considerably tighter (K_d = 2.3 mM) than L-malate (K_d = 14–33 mM; Johansson & Pettersson, 1979). Moreover, on the basis of fluorescence emission studies conducted with an extrinsic label, Kohlmann-Koch and Eggerer (1989) have reported that only oxaloacetate or analogues with the D-malate substructure induce the spectral changes suggested to indicate the open to closed conformational change. At subsaturating concentrations of L-malate, and in the absence of acetylCoA, these authors did not detect changes in fluorescence emission. Therefore, there appear to be contradictions between the results of solution studies and the crystallographic studies.

In order to address the above observations and to study the mode in which malate and the substrate acetylCoA binds to the enzyme in the ground state, four different ternary complexes were crystallized and their structures were solved by

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

[‡] Crystallographic coordinates have been submitted to the Brookhaven Protein Data Bank with designations as given in the text.

* To whom correspondence should be addressed at the Institute of Molecular Biology.

[1] Abbreviations: rms, root mean square; CoA, coenzyme A; CMCoA, carboxymethyl coenzyme A; CS, chicken heart citrate synthase.

0006-2960/91/0430-6024$02.50/0

difference Fourier map and crystallographic refinement techniques. The complexes are CS/L-malate/acetylCoA, CS/D-malate/acetylCoA, CS/L-malate/CMCoA, and CS/D-malate/CMCoA.

The crystallographic results reveal the mode of binding of acetylCoA and are consistent with proposed mechanisms (Karpusas et al., 1990; Alter et al., 1990) for the reaction. Furthermore, these studies suggest explanations, not anticipated by previous authors, for many of the physical–chemical observations discussed above.

Materials and Methods

The crystals of all four ternary complexes are isomorphous to and are grown in conditions similar to those of the ternary complexes containing citrate and CoA, citryl thioetherCoA (Remington et al., 1982), and oxaloacetate and carboxymethylCoA (Karpusas et al., 1990). The crystals are monoclinic, space group $C2$, with cell dimensions a = 104.0 Å, b = 78.1 Å, c = 58.3 Å, and β = 78.9°.

Chicken heart citrate synthase, L-malate, D-malate, and acetylCoA were purchased from Sigma. The enzyme (8 mg/mL in 0.7 M L-malate or D-malate, pH 6.0) was preincubated with either 2.5 mM acetylCoA or carboxymethylCoA for at least 1 h at room temperature. Crystallization of the different ternary complexes was accomplished by hanging drop vapor diffusion at room temperature. The drops that produced crystals consisted of 10 μL of the enzyme solution, while the wells contained approximately 1 mL of 1.1 M malate or citrate, pH 6. It was observed that the growth of crystals of adequate size (0.3 × 0.2 × 0.1 mm) required a specific salt concentration of the well solution that varied slightly depending on the enzyme stock used. Typical values were between 1.0 and 1.2 M malate or citrate, pH 6.0. No correlation was observed between the crystallization conditions and the type of ternary complex crystallized. In a few cases both "closed" monoclinic and "open" tetragonal crystals crystals grew out of the same drop [see following paper (Liao et al., 1991)].

Data were collected by oscillation photography, using a graphite monochromator (Schmidt et al., 1981), on a Rigaku RU-100 X-ray generator operated at 40 kV/130 mA. The collimator diameter was 0.4 mm. Crystals were rotated around their c axis, and photographs were taken at intervals of 2–2.5°, on Kodak No-Screen X-ray films mounted in cylindrical cassettes. The crystal to film distance was 80 mm. A program based on that of Rossmann (Rossmann, 1979; Schmid et al., 1981) was used to process the film data.

Phases derived from the isomorphous CS/oxaloacetate/CMCoA structure (Karpusas et al., 1990) were used to calculate $F_o - F_c$ and $2F_o - F_c$ difference Fourier electron density maps. These maps were used to create initial models with the program FRODO (Jones, 1978). The models were subsequently subjected to refinement with the TNT program package (Tronrud et al., 1987). In early stages of refinement the stereochemical restraints were relaxed to allow structural adjustments. Subsequently, these restraints were "tightened" to ensure good stereochemistry. Details on the data collection and the refinement statistics are summarized in Table I. Atomic coordinates have been deposited in the Brookhaven Protein Data Bank (Bernstein et al., 1977). The data bank designations for these coordinates are CS/L-malate/CMCoA, 1CSC; CS/D-malate/CMCoA, 2CSC; CS/L-malate/acetylCoA, 3CSC; CS/D-malate/acetylCoA, 4CSC.

Results and Discussion

In all four complexes the structure of the enzyme is virtually identical with that of the previously determined monoclinic structures. The largests differences are on the order of 0.5 Å for some side chains in direct contact with the substrate analogues. In addition, the adenine nucleotide and pantothenate moieties of the coenzyme A analogue assume identical conformations within the estimated error of these structure determinations (estimated to be between 0.1 and 0.2 Å rms).

Table I: Experimental Data and Summary of Atomic Models

space group	$C2$			
cell dimensions	a = 104.1 Å, b = 78.1 Å, c = 58.3 Å, β = 78.9°			
	model			
	L-Mal/CMCoA	D-Mal/CMCoA	L-Mal/AcCoA	D-Mal/AcCoA
Refinement Statistics				
no. of films	39	36	34	37
observations	67 588	52 144	48 525	51 703
unique reflections	32 414	25 075	23 641	24 109
R_{merge}[a] (%)	7.6	9.8	10.6	9.7
resolution (Å)	1.7	1.9	1.9	1.9
R factor[b] (%)	18.8	18.9	17.7	18.8
Deviations from Ideality (rms)				
bonds (Å)	0.018	0.103	0.020	0.013
angles (deg)	3.7	2.6	2.6	2.5
planarity (Å)	0.035	0.026	0.022	0.024
bad contacts (Å)	0.096	0.052	0.066	0.033

[a] R_{merge} gives the average disagreement in percent for repeated measurements of an intensity. [b] R factor is the standard crystallographic reliability factor.

Table II: Selected Temperature Factors (Å²)[a]

	model			
atom	L-Mal/CMCoA	D-Mal/CMCoA	L-Mal/AcCoA	D-Mal/AcCoA
O91	11.85	19.24	38.02	31.89
O92/C92	16.92	22.73	46.64	38.63
C91	21.53	23.52	33.42	28.27
C81	14.13	11.94		
S81	10.01	15.30	32.90	52.48

[a] These demonstrate the increased mobility of the acetyl group of acetylCoA over that of the carboxymethyl group of carboxymethylCoA.

Ternary Complexes with AcetylCoA. (i) Binding of Acetyl Coenzyme A. There is observable electron density for the ground-state conformation of the acetyl group of acetylCoA in the CS/L-malate/acetylCoA and CS/D-malate/acetylCoA structures (Figures 1 and 2). The acetyl group is in a somewhat different conformation in each case (Figure 2), possibly due to differing steric interactions with D- or L-malate. Although the plane of the acetyl group is clear, the electron density is not as well-defined as in the cases of the structures with CMCoA discussed below and is consistent with some disorder, either dynamic or static with multiple conformations, of the acetyl group. The high temperature factors of the atoms of the acetyl group are consistent with either of these possibilities (see Table II). In the following discussion we make note of distances and angles between atoms of the acetyl group and those of the protein or malate, but we stress that these are only approximate as the refined model represents a compromise between one or more (possibly disordered) conformations of the acetyl group.

It is not possible on the basis of these density maps to distinguish the methyl group from the carbonyl oxygen of the acetyl group, but in both instances there is density for an assumed water molecule (585 OH) with reasonable hydrogen-bonding distance and geometry from the "lower" atom in Figure 1, panels a and b. This atom is in a position to make

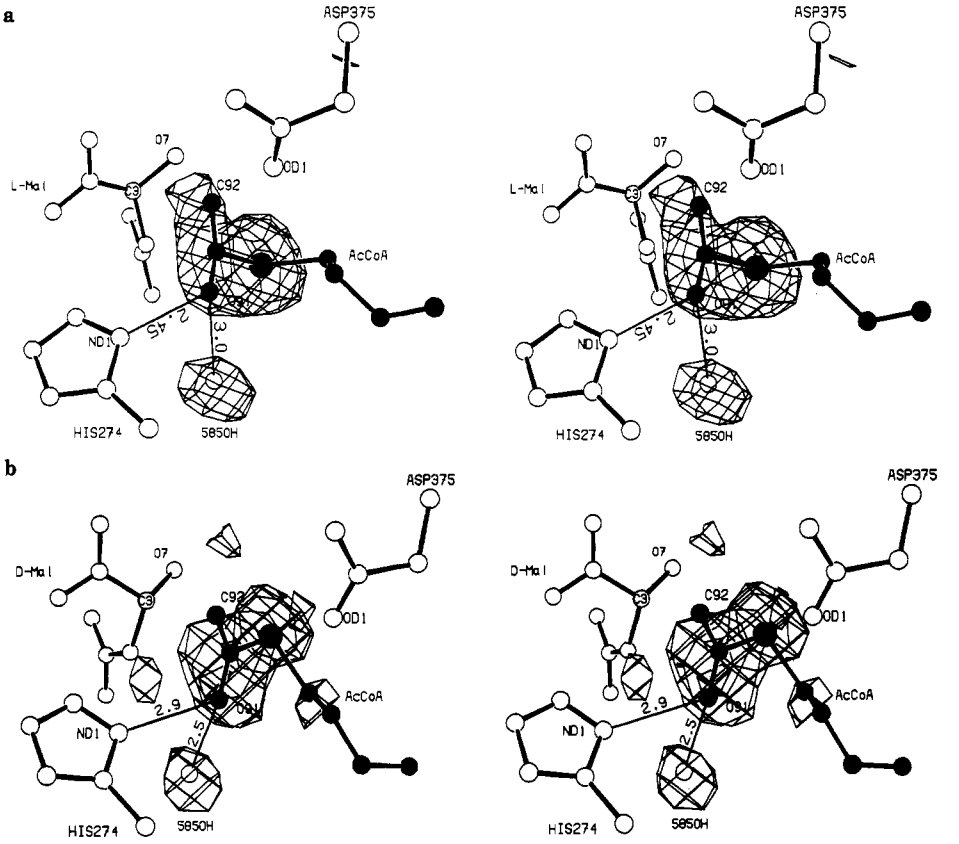

FIGURE 1: (a) Stereoview of an $F_o - F_c$ difference electron density map contoured at 1 standard deviation for the CS/L-malate/acetylCoA complex. The final model for the CS/L-malate/CMCoA complex, from which the atoms of the carboxymethyl group and sulfur S81 were removed, was used for the calculation of phases. The final refined model of the CS/L-malate/acetylCoA complex is shown superimposed. The acetylCoA atoms and bonds are filled, and the sulfur is drawn with a radius twice that of the lighter atoms. Possible hydrogen bonds of the oxygen of the acetyl group to His 274 ND1 and a presumed water molecule (585 OH) are shown as thin bonds with the indicated lengths in Å. (b) As in (a), stereoview of an $F_o - F_c$ difference electron density map contoured at 1 standard deviation for the CS/D-malate/acetylCoA complex. The refined model for the CS/D-malate/CMCoA complex, from which the atoms of the carboxymethyl group and sulfur S81 were removed, was used for the calculation of phases. As discussed in the text, there appears to be some disorder of the acetyl group, and the refined model represents a compromise. This is evident from the fact that the acetyl group does not fit the contour cage as well as one would like.

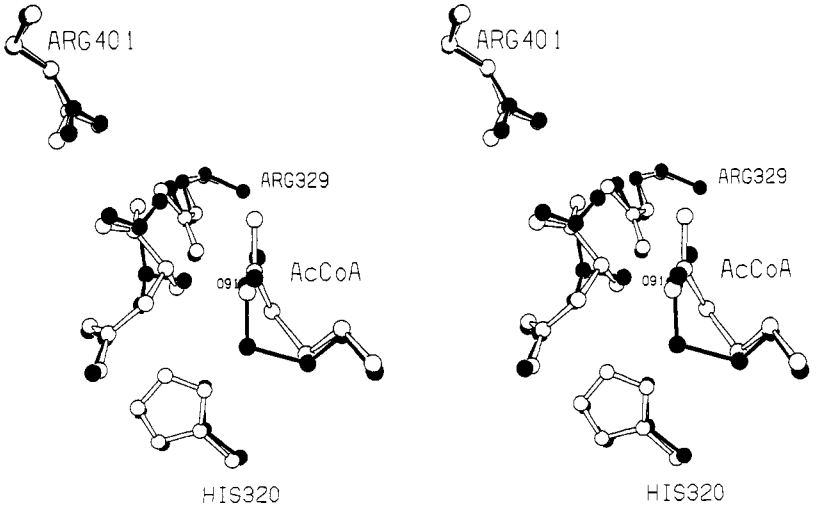

FIGURE 2: Final refined model of the complex CS/D-malate/acetylCoA complex (open bonds and atoms) superimposed on the refined model of the CS/L-malate/acetylCoA complex (filled bonds and atoms). Drawing shows conformational adjustments of protein side chains which accommodate the differing stereochemistry of the malate isomers. In addition, the average conformation of the acetyl group of acetylCoA is seen to differ in the two complexes.

a good hydrogen bond to His 274 ND1 as well, so we interpret this to be the carbonyl oxygen of the acetyl group (O91 in Figures 1 and 2). This is in keeping with our previous argument (Karpusas et al., 1990) that His 274 is most likely the proton donor during the enolization reaction. The methyl group is in tight van der Waals contact (approximately 2.9 Å) with both the C3 carbon of malate and OD1 of aspartate 375, but would not in be in an optimal position to hydrogen bond to the latter residue if this were the carbonyl oxygen. Upon comparison (Figure 2) of both structures, one notes that the carbonyl oxygen occupies approximately the same position in both "average" conformations of the acetyl group but the other atoms are free to move, again suggesting the importance of the two hydrogen bonds to this atom.

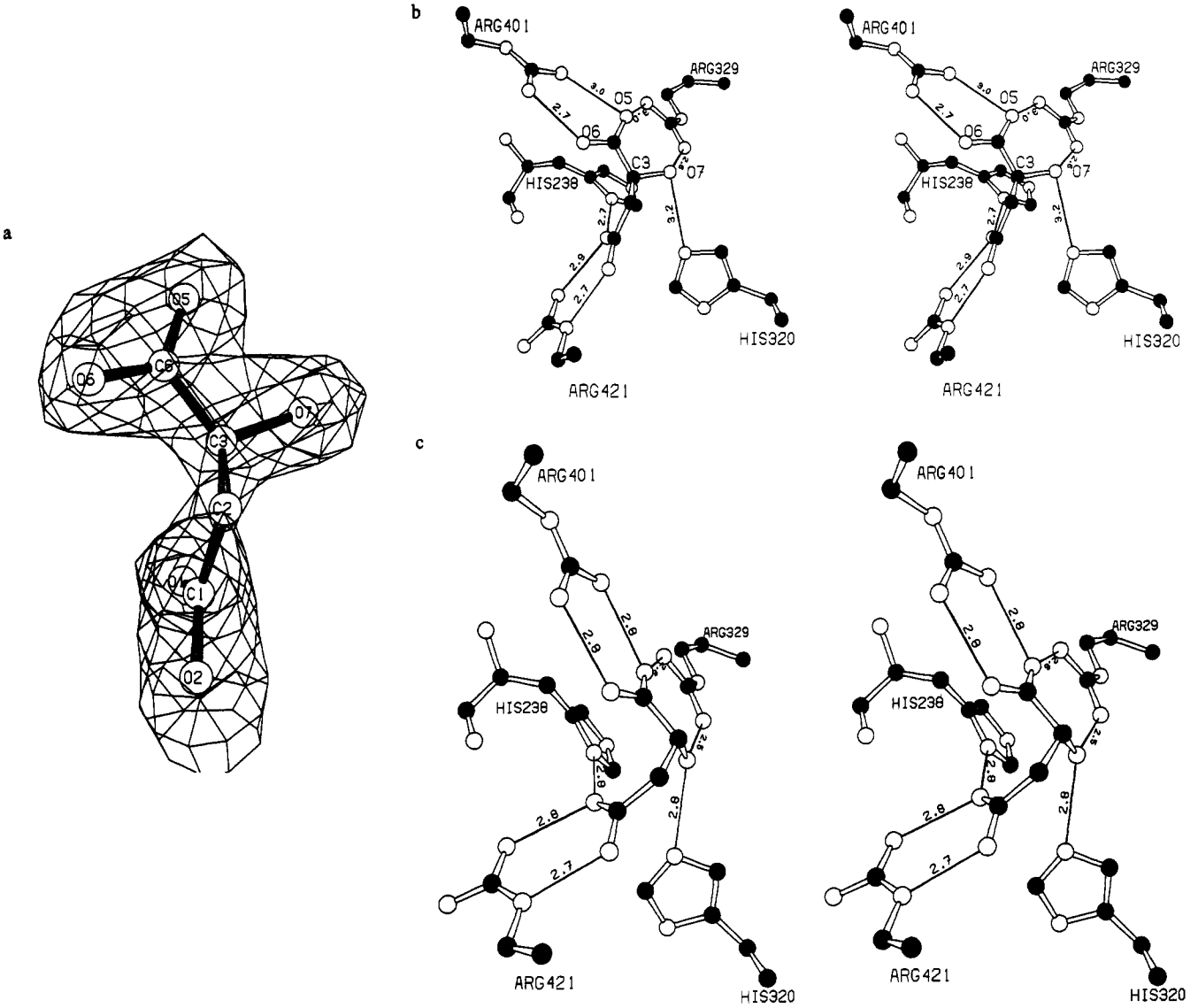

FIGURE 3: (a) Final 1.9-Å $2F_o - F_c$ electron density map of the CS/L-malate/acetylCoA complex contoured at 1 standard deviation about the L-malate molecule. This is representative of the clarity of the four electron density maps for well-ordered regions of the molecule and shows that the orientation of bound malate is unambiguous. (b) Detailed conformation of bound L-malate in the CS/L-malate/acetylCoA complex. The thin bonds represent hydrogen bonds or salt bridges to protein atoms with the indicated lengths in Å. Carbon atoms are filled while nitrogen and oxygen atoms are unfilled. (c) As in (b) showing detailed conformation of bound D-malate in the CS/D-malate/acetylCoA complex.

(ii) Binding of D- *and* L-*Malate.* The electron density maps reveal the conformation of bound D- and L-malate in a clear and convincing manner (see Figure 3a for a representative case). A striking feature of these maps is that both D- and L-malate bind to the enzyme identically in fashion to the binding of oxaloacetate and citrate in complexes previously studied [Remington et al., 1982; Wiegand et al., 1984; Figure 2 of Karpusas et al. (1990)], where the oxygens O1, O2, O5, O6, and O7 form hydrogen bonds or salt bridges with the same protein atoms as in the case of oxaloacetate or citrate (see Figure 3). Small but concerted shifts on the part of side chains His 274, His 320, Arg 329, Asp 375, and Arg 401 (see Figure 2) take place to compensate for the different geometry of the two inhibitors. The rather different binding constants of these two stereoisomers must be a consequence of the hydrogen bond geometry rather than their number. Both L-malate and D-malate make 8 hydrogen bonds with the same protein atoms, but the binding affinity of D-malate is higher than that of L-malate by at least 1 order of magnitude as previously noted. The hydrogen bond geometry is summarized in Table III and Figure 3b,c. As can be seen, the major differences concern the geometry of interactions at the O7 hydroxyl of malate. In particular, the H–N–O angle (atomic coordinates were calculated for the hydrogen atom based on the positions of the heavier atoms), which should be in the vicinity of 0° (Baker & Hubbard, 1984), is substantially larger in several instances for L-malate than in D-malate as are several of the hydrogen bond lengths. It is not unreasonable to attribute the difference

Table III: Selected Hydrogen Bond Angles (deg)[a]

atoms defining angle[b]	model: L-Mal/AcCoA	model: D-Mal/AcCoA
H-320NE2-702O7	31.7	21.6
H-329NH2-702O7	13.6	18.6
H-329NH1-702O5	7.1	4.8
H-401nh1-702O5	6.7	2.2
H-401NH2-702O6	19.5	3.7

[a] The H–N–O angle should be small in an ideal hydrogen bond. Here, it is seen that L-malate appears to form poorer hydrogen bonds than does D-malate. [b] 320, 329, 401, and 702 are the residue numbers of His 320, Arg 329, Arg 401, and D- or L-malate, respectively. Hydrogen coordinates were calculated based on heavy atom positions.

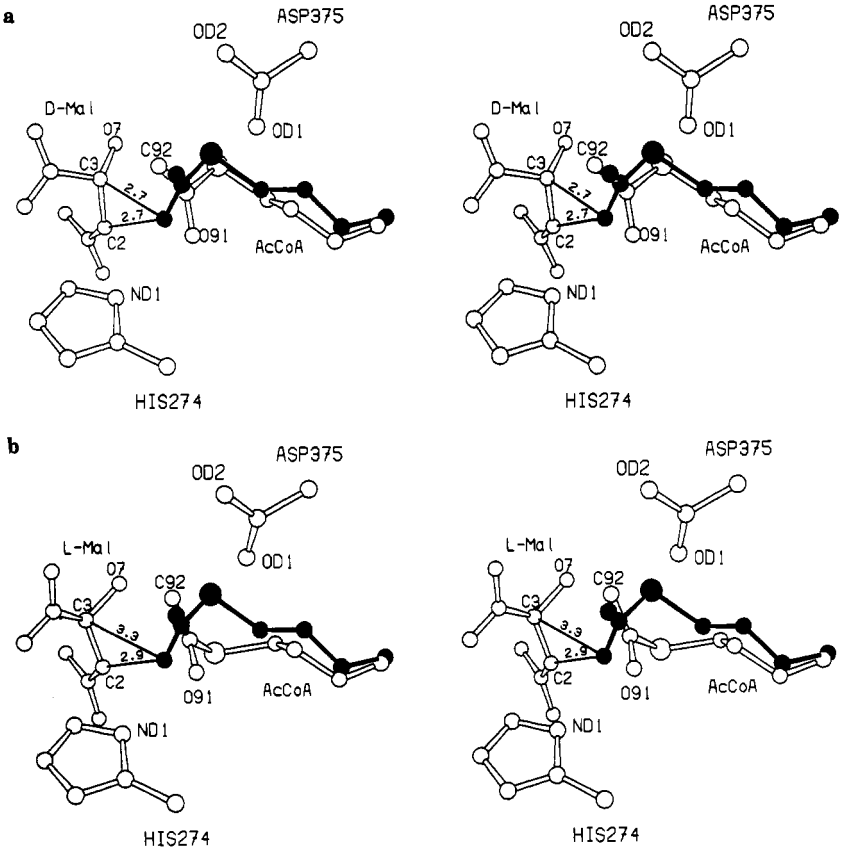

FIGURE 4: (a) Hypothetical transition-state model for the enolization of acetyl-CoA (filled bonds and atoms) superimposed on the observed model CS/D-malate/acetylCoA (open bonds and atoms). The closest contact distances of O91 of the transition-state model with atoms of D-malate are indicated by thin bonds and have the indicated lengths in Å. (b) As in (a), but the observed model is the CS/L-malate/acetylCoA (open bonds and atoms). The shortest contact distances of the O91 of the hypothetical enolization transition state of acetylCoA with atoms of L-malate are indicated by thin bonds and have the indicated lengths in Å.

in affinity to these differences in bond geometry; however, there may also be some steric interference as well.

(*iii*) *Steric Interactions of Malate and Acetyl Coenzyme A.* Due to the difference in the chirality of L- and D-malate the C3 carbon lies on different sides of the plane of the atoms C2, O7, and C6, the three of which occupy similar positions when bound to the enzyme. An obvious consequence of this difference is that the conformational flexibility of acetylCoA may be more restricted when D-malate is bound rather than L-malate, due to reduced space in the active site. To visualize the effects of this difference, we have superimposed each of the CS/L-malate/acetylCoA and CS/D-malate/acetylCoA models with the hypothetical model for the transition state of the enolization reaction (the coordinates of the methyl group and the carbonyl oxygen of acetylCoA are those observed for the carboxylate oxygens of carboxymethylCoA; Karpusas et al., 1990). The superimpositions show that the acetyl group in the hypothetical transition state is somewhat rotated and displaced with respect to the position in the ground state (Figure 4). Figure 4a shows that in the case of the complex with D-malate the carbonyl oxygen of acetylCoA (O91) in the proposed transition-state conformation would lie about 2.7 Å from each of the C3 and C2 atoms of D-malate. In the case of L-malate the same distances would be about 3.2 and 2.9 Å, respectively (Figure 4b). This suggests that the lack of observable tritium incorporation into acetylCoA in the presence of D-malate may be a consequence of the fact that the acetyl group cannot easily assume the transition-state conformation due to steric hindrance with D-malate. In the case of L-malate there would be less steric hindrance and thus acetylCoA could undergo enolization and proton exchange. Although 2.9 Å would be a rather bad contact, this is consistent with the fact that the reaction is very slow. Incubation times of many hours are required to detect a reasonable incorporation of tritium label into acetylCoA in the presence of L-malate (Eggerer, 1965). Presumably proton exchange can occur in the presence of D-malate, but is so slow as to be indistinguisable from background.

Ternary Complexes with Carboxymethyl Coenzyme A. (*i*) *Binding of Carboxymethyl Coenzyme A.* The carboxymethyl group of CMCoA in both CS/L-malate/CMCoA and CS/D-malate/CMCoA forms two hydrogen bonds with His 274 and Asp 375 in the same manner as in the CS/oxaloacetate/CMCoA structure (Karpusas et al., 1990). This group is very clearly defined in the electron density map, indicating that it is rigidly bound. In addition, the vibrational parameters of the carboxymethyl moiety average about 14 Å^2 (Table III), which is comparable to those of the most highly ordered interior atoms of the protein. This is rather different than the conformation assumed by the acetyl group when acetylCoA is bound as discussed above. This is not an anticipated result and, at first glance, appears to contradict our arguments about steric clashes between malate and acetylCoA. However, inspection of the conformations of bound malate reveals both stereoisomers to be bound in a less favorable fashion than is the case with acetylCoA. Evidently, a compromise has been established between the binding modes of the two substrate analogues.

(*ii*) *Binding of* D- *and* L-*Malate.* The CS/L-malate/CMCoA structure provides a surprising observation: L-malate binds in a very different conformation from that in the CS/L-malate/acetylCoA structure. Although a carboxyl group (atoms C1, O1, and O2) and atom C2 maintain analogous positions in both structures, the rest of the molecule is rotated

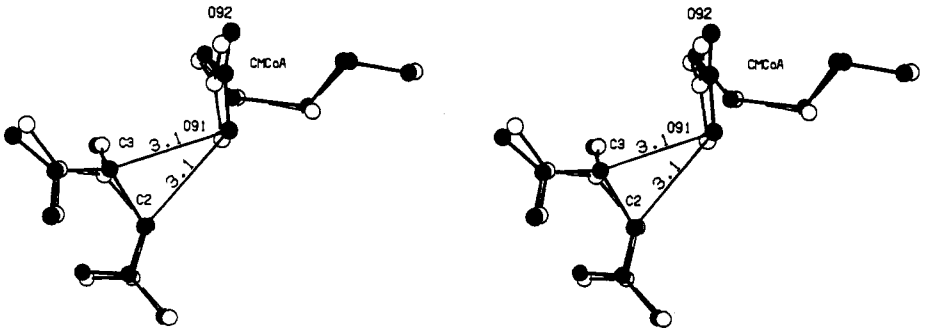

FIGURE 5: Stereoview of a comparison of two complexes containing carboxymethylCoA. Open bonds and atoms are from the CS/oxaloacetate/CMCoA model of Karpusas et al. (1990). Filled bonds and atoms are from the CS/D-malate/CMCoA model and show that both D-malate and the carboxymethyl group shift away from their mean positions in the oxaloacetate complex. The cause is evidently the steric clash indicated by the thin bonds, which have the indicated distance in Å.

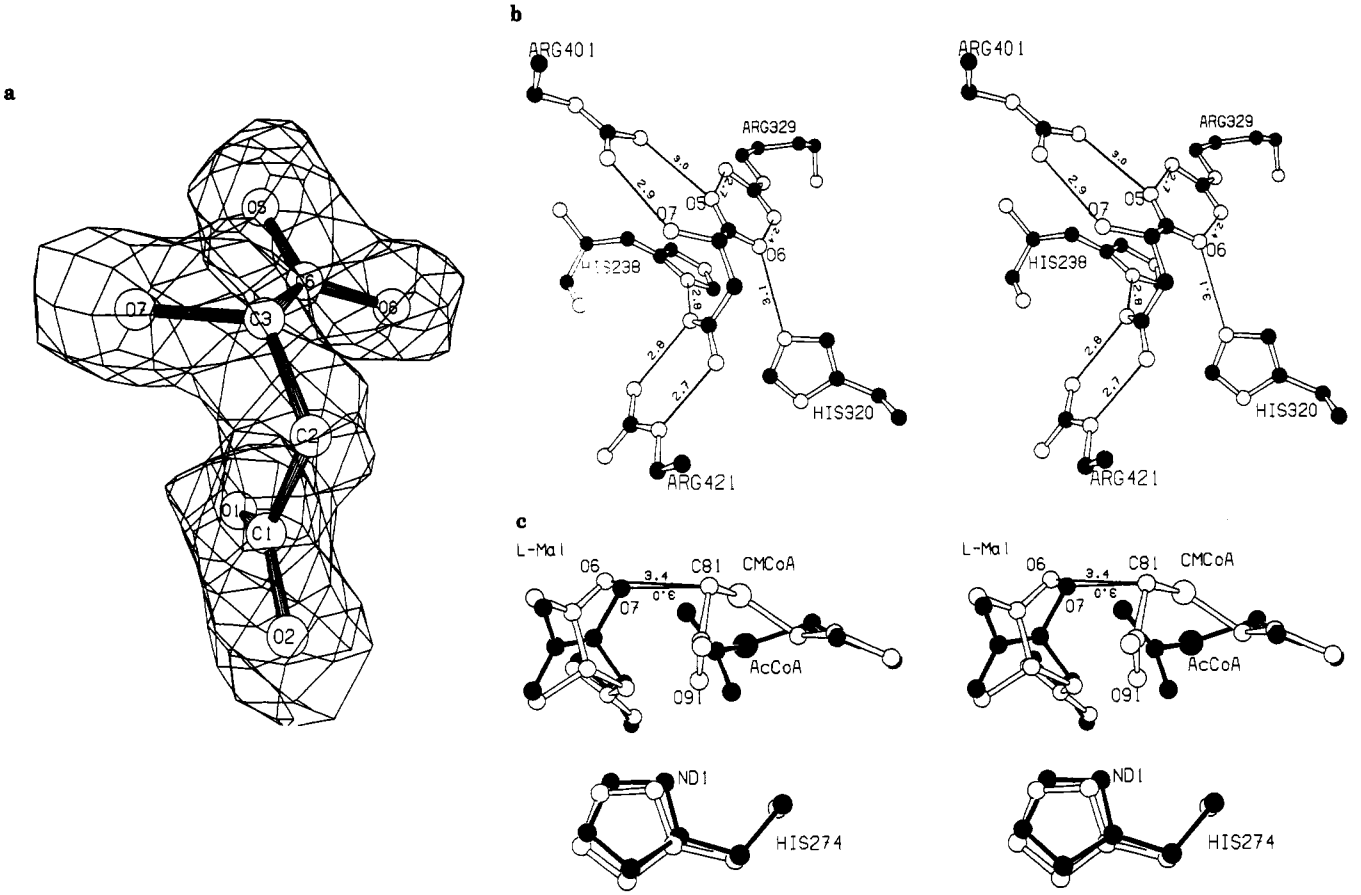

FIGURE 6: Alternate binding of L-malate in the CS/L-malate/carboxymethylCoA complex. (a) Final $2F_o - F_c$ electron density map contoured at 1 standard deviation showing the conformation of L-malate. The map shows that the conformation is unambiguous. (b) Stereo diagram showing the detailed conformation and hydrogen bonding of L-malate. Conventions are as in Figure 3. (c) Superimposition of the refined models for the CS/L-malate/CMCoA complex (open bonds and atoms) and CS/L-malate/acetylCoA complex (filled bonds and atoms). The shortest contact distances of the C81 of CMCoA to the nearest atom of L-malate in either conformation of L-malate are plotted as thin bonds nad have the observed (open bonds) or hypothetical (filled bonds) lengths in Å.

by about 180° around the C2–C3 bond (Figure 6). Superimposition of the CS/L-malate/acetylCoA and CS/L-malate/CMCoA structures shows that the protein residues (His 320, His 238, Arg 401, Arg 421, and Arg 329) involved in the binding of L-malate maintain the same positions (within the limits of accuracy of the crystal structures); therefore, this second mode binding is not a consequence of small conformational changes of the enzyme. In contrast, D-malate binds in essentially the same manner to the enzyme regardless of which CoA analogue is present, except that the molecule is driven about 0.2–0.3 Å deeper into the binding pocket, apparently due to steric interactions with the carboxylate of CMCoA.

(iii) Steric Interactions of Malate with Carboxymethyl Coenzyme A. In the initial stages of the analysis of the malate/CMCoA structures, phases were derived from the refined model of the CS/oxaloacetate/CMCoA complex (Karpusas et al., 1990) and $F_o - F_c$ difference electron density maps were calculated. Difference features were apparent due to the differences between malate and oxaloacetate. However, the maps also revealed a shift of the carboxymethyl group of CMCoA about 0.2 Å away from its position in the oxaloacetate complex (Figure 5). As stated above, D-malate shifts when CMCoA is substituted for acetylCoA, and these shifts are in opposite directions. Thus, it appears that there is steric strain in both complexes of malate with CMCoA, which is

accommodated by two different mechanisms. In the case of D-malate, small shifts appear to be sufficient to relieve bad contacts; however, this does not appear to be possible for L-malate, which binds in an alternative mode. As seen in Figure 5, the shortest contact between CMCoA and D-malate or oxaloacetate is 3.1 Å in both cases, which is apparently the limit tolerated.

Figure 6 shows that the closest contact between L-malate and CMCoA is between C81 of CMCoA (this atom is not present in acetylCoA) and O6 of L-malate. These atoms are separated by 3.4 Å (unfilled atoms and bonds). Superimposed in this figure is the L-malate conformation observed in the CS/L-malate/acetylCoA complex (filled atoms and bonds). The distance between C81 of CMCoA and O7 of L-malate in this conformation would be 3.0 Å, which is quite short. We suggest that the different mode of binding of L-malate is a consequence of this short contact. L-Malate appears to be "forced" to bind in a different, almost certainly less favorable mode than that observed in the CS/L-malate/acetylCoA complex. Model building shows that there is enough space for L-malate to bind in either mode in the CS/L-malate/acetylCoA complex. However, the electron density map shows no evidence for such mixed binding, which again supports the notion that this is the energetically preferred mode.

Conclusions

The isomorphous crystal structures of the four ternary complexes of D- and L-malate with acetyl coenzyme A or carboxymethyl coenzyme A have been determined and fully refined at 1.9-Å resolution.

The complexes with acetylCoA reveal the carbonyl of the acetyl group to be hydrogen bonded to His 274 and a water molecule, while the methyl group is in van der Waals contact with Asp 375, which has been suggested to be the base in the enolization reaction. The complexes support the proposals for the concerted acid/base roles of His 274 and Asp 375 (Karpusas et al., 1990; Alter et al., 1990). These complexes also reveal that D- and L-malate bind in a fashion similar to oxaloacetate and make the same interactions with hydrogen-bonding groups on the enzyme. However, the hydrogen bonds tend to be less optimal in geometry for L-malate than for D-malate, which may account for the 10-fold lower affinity of L-malate for the enzyme. The conformations of bound malate also suggest an explanation for the puzzling observation that citrate synthase slowly catalyzes exchange of protons of acetylCoA with solvent in the presence of L-malate, but not D-malate (Eggerer, 1965). Steric hindrance with D-malate, but not L-malate, would seem to prevent the acetyl group from attaining the transition-state configuration of the enolization reaction (at a measurable rate) as suggested in Figure 4.

The complexes with carboxymethyl coenzyme A and D- or L-malate suggest that there is considerable steric strain between these two substrate analogues. D-Malate is forced deeper into the binding pocket when CMCoA replaces acetylCoA, and the carboxymethyl group is forced away from the position it occupies in the oxaloacetate/carboxymethyl CoA complex. L-Malate is forced to bind in an alternative mode to that seen in the complex with acetylCoA, presumably because bad contacts with the "extra" methylene group of CMCoA would take place at the O7 hydroxyl of L-malate in the preferred binding mode. We argue that the strong binding of CMCoA compensates energetically for a less favorable mode of binding of L-malate and offsets steric strain in the case of D-malate. CMCoA is known to bind to citrate synthase at least 3000 times more tightly than acetylCoA and for this reason was suggested to be a transition-state analogue (Bayer et al., 1981). We make the testable prediction that binding energy of carboxymethylCoA compensates for the bond distortions that seem to be required to form the ternary complexes. This underscores the suggestion that *citrate synthase is optimally configured to bind the transition state of the enolization reaction, which is the rate-limiting step of the overall reaction.* If necessary, other substrate analogues are forced to bind in suboptimal configurations to accommodate the transition-state analogue.

A recent study [Liao et al., 1991 (following paper)] suggests that in solution there is an equilibrium between the open and closed conformations of citrate synthase, with the equilibrium constant depending on the strength of the binding of the ligand(s) present. If ligands of large binding constant are present, then the equilibrium favors the closed conformation. The observed conformation in these crystal structures with both D- and L-malate and acetylCoA is the closed one. Contrary to the suggestions of Kollmann-Koch and Eggerer (1989), this conformation therefore exists in solution in the presence of acetylCoA and L-malate. It is possible that acetylCoA in addition to L-malate is required to form a significant equilibrium level of this conformation in solution. This would be consistent with the conclusions of Johansson and Pettersson (1979) that the acetyl group contributes significantly to binding in the ternary complexes. While oxaloacetate binds to citrate synthase noncooperatively with CoA or ATP, it shows a 20-fold increase in affinity in the presence of acetylCoA. Furthermore, both L- and D-malate bind to the close conformation in the same fashion in the presence of acetylCoA. Therefore, explanations for the differing catalytic activity of the two complexes must lie elsewhere as we have discussed.

Acknowledgments

We thank Dr. Linda Kurz for a generous gift of carboxymethyl coenzyme A used in this investigation and Tim Bullock for technical assistance.

References

Alter, G., Casazza, J., Zhi, W., Nemeth, P., Srere, P. A., & Evans, C. T. (1990) *Biochemistry 29*, 7557–7563.

Baker, E. N., & Hubbard, R. E. (1984) *Prog. Biophys. Mol. Biol. 44*, 97–179.

Bayer, E., Bauer, B., & Eggerer, H. (1981) *Eur. J. Biochem. 120*, 47–52.

Beeckmans, S. (1984) *Int. J. Biochem. 16*, 341–351.

Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, E. F., Jr., Brice, M. D., Rodgers, J. R., Kennard, O., Schimanouchi, T., & Tasunmi, M. (1977) *J. Mol. Biol. 112*, 535–542.

Eggerer, H. (1965) *Biochem. Z. 343*, 111–138.

Eggerer, H., Buckel, W., Lenz, H., Wunderwald, P., Cornforth, J. W., Donninger, C., Mallaby, R., Redmond, J. W., & Gottschalk, G. (1970) *Nature (London) 226*, 517–519.

Johansson, C.-J., & Pettersson, G. (1979) *Eur. J. Biochem. 93*, 505–513.

Jones, T. A. (1978) *J. Appl. Cryst. 11*, 268–272.

Karpusas, M., Branchaud, B., & Remington, S. J. (1990) *Biochemistry 29*, 2213–2219.

Kollmann-Koch, A., & Eggerer, H. (1989) *Eur. J. Biochem. 185*, 441–447.

Liao, D.-I., Karpusas, M., & Remington, S. J. (1991) *Biochemistry* (following paper in this issue).

Remington, S., Wiegand, G., & Huber, R. (1982) *J. Mol. Biol. 158*, 111–152.

Rossmann, M. (1979) *J. Appl. Crystallogr. 12*, 225–238.

Schmid, M. F., Weaver, L. H., Holmes, M. A., Gruetter, M. G., Ohlendorf, D. H., Reynolds, R. A., Remington, S. J., & Matthews, B. W. (1981) *Acta Crystallogr. A37*, 701–710.
Srere, P. (1966) *J. Biol. Chem. 241*, 2157–2165.
Srere, P. (1972) *Curr. Top. Cell. Regul. 5*, 245–283.
Tronrud, D. E., Ten Eyck, L. F., & Matthews, B. W. (1987) *Acta Crystallogr. A43*, 489–501.
Weitzman, P. D. J., & Danson, M. J. (1976) *Curr. Top. Cell. Regul. 10*, 161–204.
Wiedman, S. W., Drysdale, G. R., & Mildvan, A. S. (1973) *Biochemistry 12*, 1874–1883.
Wiegand, G., & Remington, S. (1986) *Annu. Rev. Biophys. Biophys. Chem. 15*, 97–117.
Wiegand, G., Remington, S., Deisenhofer, J., & Huber, R. (1984) *J. Mol. Biol. 174*, 205–211.

Crystal Structure of an Open Conformation of Citrate Synthase from Chicken Heart at 2.8-Å Resolution[†,‡]

Der-Ing Liao, Mihail Karpusas, and S. James Remington*
Institute of Molecular Biology and Department of Physics, University of Oregon, Eugene, Oregon 97403
Received October 12, 1990; Revised Manuscript Received February 22, 1991

ABSTRACT: The X-ray structure of a new crystal form of chicken heart muscle citrate synthase, grown from solutions containing citrate and coenzyme A or L-malate and acetyl coenzyme A, has been determined by molecular replacement at 2.8-Å resolution. The space group is $P4_3$ with $a = 58.9$ Å and $c = 259.2$ Å and contains an entire dimer of molecular weight 100 000 in the asymmetric unit. Both "closed" conformation chicken heart and "open" conformation pig heart citrate synthase models (Brookhaven Protein Data Bank designations 3CTS and 1CTS) were used in the molecular replacement solution, with crystallographic refinement being initiated with the latter. The conventional crystallographic R factor of the final refined model is 19.6% for the data between 6- and 2.8-Å resolution. The model has an rms deviation from ideal values of 0.034 Å for bond lengths and of 3.6° for bond angles. The conformation of the enzyme is essentially identical with that of a previously determined "open" form of pig heart muscle citrate synthase which crystallizes in a different space group, with one monomer in the asymmetric unit, from either phosphate or citrate solution. The crystalline environment of each subunit of the chicken enzyme is different, yet the conformation is the same in each. The open conformation is therefore not an artifact of crystal packing or crystallization conditions and is not species dependent. Both "open" and "closed" crystal forms of the chicken heart enzyme grow from the same drop, showing that both conformations of the enzyme are present at equilibrium in solution containing reaction products or substrate analogues.

In higher organisms citrate synthase is a dimer of two identical subunits related by a 2-fold axis and has a total molecular weight of about 100 000. For extensive reviews of the physical–chemical properties of the enzyme from a wide variety of sources, see Srere (1972), Weitzman and Danson (1976), Beeckmans (1984), and Wiegand and Remington (1986). The structures of three different crystal forms of pig and chicken heart muscle citrate synthase have previously been determined [Remington et al., 1982; Wiegand et al., 1984; Karpusas et al., 1990, 1991 (preceding paper in this issue)] for unliganded enzyme and a variety of ternary and binary complexes. Each subunit can be described as consisting of two domains, a large and a small one, with the substrate binding site in the cleft between the two domains. The enzyme was the first shown to consist almost entirely of α helices.

Citrate synthase from pig heart has been shown to be capable of very large conformational changes. Two of the crystal structures (space groups $C2$ and $P4_32_12$) are very similar and are classified as "closed" (Remington et al., 1982; Wiegand et al., 1984; Wiegand & Remington, 1986). The two closed forms differ primarily in the arrangement of internal side chains while the third one (space group $P4_12_12$) is "open" (Remington et al., 1982).

In the "closed" forms ligands are buried inside the cleft and inaccessible to bulk solvent. The change in conformation between the open and closed form can be roughly approximated by a rigid body rotation of 19° of the small domain relative to the large one; however, there are substantial changes in the packing of side chains in the interior of the small subunit (Wiegand et al., 1984; Lesk & Chothia, 1984) upon the transition between conformations. Lesk and Chothia have argued that the conformational change is best described as a concerted series of shifts between adjacent helices (a "plastic" deformation) rather than a rigid body rotation. This description implies that a continuous range of stable conformations may be available to a given molecule, which we feel may be incorrect and additionally yield a misleading picture as to the mechanism of conformational change. In this instance, we present evidence which suggests that there are only two stable conformational states of citrate synthase relevant to the enzymatic mechanism.

Evidence for conformational change upon addition of ligands has been obtained from a variety of spectroscopic studies of the protein in solution (Srere, 1966; Bayer et al., 1981; Weidman et al., 1981; Kollmann-Koch & Eggerer, 1989). These and crystallographic studies suggest that the binding

[†] This research was supported in part by a grant from the National Science Foundation (DMB-8817438) to S.J.R. and a grant to the Institute of Molecular Biology from the Lucille P. Markey Charitable Trust.
[‡] Crystallographic coordinates have been submitted to the Brookhaven Protein Data Bank with designations as given in the text.
* To whom correspondence should be addressed at the Institute of Molecular Biology.

0006-2960/91/0430-6031$02.50/0