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Aetyl-CoA Enolization in Citrate Synthase: A Quantum Mechanical/Molecular Mechanical (QM/MM) Study

Adrian J. Mulholland[1] **and W. Graham Richards**[1]*
[1]*Physical and Theoretical Chemistry Laboratory, Oxford University, Oxford, United Kingdom*

ABSTRACT **Citrate synthase forms citrate by deprotonation of acetyl-CoA followed by nucleophilic attack of this substrate on oxaloacetate, and subsequent hydrolysis. The rapid reaction rate is puzzling because of the instability of the postulated nucleophilic intermediate, the enolate of acetyl-CoA. As alternatives, the enol of acetyl-CoA, or an enolic intermediate sharing a proton with His-274 in a "low-barrier" hydrogen bond have been suggested. Similar problems of intermediate instability have been noted in other enzymic carbon acid deprotonation reactions. Quantum mechanical/molecular mechanical calculations of the pathway of acetyl-CoA enolization within citrate synthase support the identification of Asp-375 as the catalytic base. His-274, the proposed general acid, is found to be neutral. The acetyl-CoA enolate is more stable at the active site than the enol, and is stabilized by hydrogen bonds from His-274 and a water molecule. The conditions for formation of a low-barrier hydrogen bond do not appear to be met, and the calculated hydrogen bond stabilization in the reaction is less than the gas-phase energy, due to interactions with Asp-375 at the active site. The enolate character of the intermediate is apparently necessary for the condensation reaction to proceed efficiently. Proteins 27:9–25**

Key words: CHARMM; enzyme reaction intermediate; strong hydrogen bonds; Marcus formalism analysis

INTRODUCTION

To understand the mechanisms of enzymes, and account for the rapid rates of reaction they typically exhibit, the precise roles of catalytic residues must be determined, any reaction intermediates identified, and significant interactions quantified. Computer simulation is an attractive approach for the study of enzyme-catalyzed reactions.[1] Studies of this type aim to calculate the energy profile for the reaction within the enzyme directly, and can provide information which is often inaccessible experimentally, for example, on unstable species and transition states (TSs), and contributions to catalysis. For calculations on reactions in enzymes to be reliable, the effects of the environment on the reaction must be included. This can be achieved with methods that combine a quantum mechanical (QM) model of the reactive system with a simpler molecular mechanics (MM) description of the protein surroundings.[2,3]

A large and important class of enzyme-catalyzed reactions involve deprotonation of a carbon atom adjacent to a carbonyl group, for which it has proved particularly difficult to characterize the mechanisms and explain the observed reaction rates, despite the availability of detailed structural and kinetic data in many instances. Discussion has centered on the nature of the reaction intermediate, if any, formed by deprotonation of the weakly acidic carbon acid substrates. The charged (enolate) intermediates proposed for many reactions of this type[4] have been suggested to be too unstable to be compatible with the rapid reaction rates unless significantly stabilized by the enzyme.[5] Concerted mechanisms avoiding the charged intermediate, for example, the formation of a neutral enol form of a substrate by concerted acid-base catalysis, have been proposed as alternatives. Stabilization of charged intermediates and TSs in these and other enzymic reactions has been attributed to "short, strong" or "low-barrier" hydrogen bonds.[6,7] This proposal has been the subject of widespread debate, and the strength of such interactions in proteins remains uncertain.[8]

Examples of enzymes of this class include triosephosphate isomerase (TIM), mandelate racemase, 3-oxo-Δ^5-steroid isomerase, and citrate synthase. Citrate synthase (CS) catalyzes the formation of citrate from acetyl-coenzyme A (CoA) and oxaloacetate (Fig. 1), the first step of the citric acid cycle. CSs from eukaryotes, the archaea, and gram-positive bacteria are homodimeric, whereas those from gram-negative bacteria are homohexamers.[9] CS has been studied extensively for many years by a

Dr. Mulholland's current address is Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge MA 02138, U.S.A.
*Correspondence to: Dr. W. Graham Richards, Physical and Theoretical University Laboratory, Oxford University, South Parks Road, Oxford, United Kingdom.
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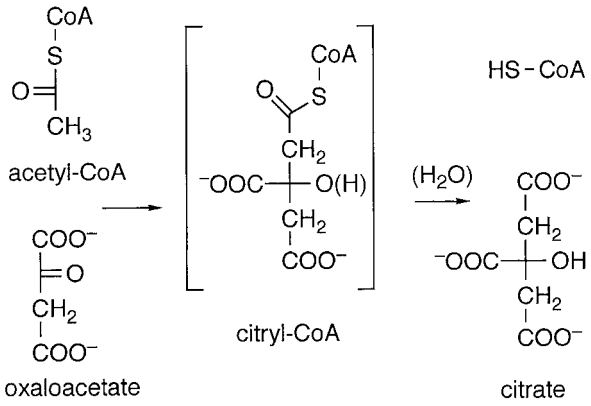


Fig. 1. The citrate synthase reaction.

variety of techniques. The enzymes from pig and chicken are particularly well characterized. High-resolution crystal structures of these two very similar CSs have been solved alone and with a variety of inhibitors and substrates bound.[8,10]

The reaction of acetyl-CoA with oxaloacetate is a Claisen-type condensation, and occurs with inversion of configuration at the acetyl methyl carbon.[11,12] In the majority of CSs (strictly designated *S*-citrate synthases), addition is to the *si* face of oxaloacetate; the rare *R*-CS is mechanistically distinct.[13] Oxaloacetate is activated towards nucleophilic attack by polarization at the active site.[14] Citryl-CoA is formed as an intermediate by the condensation reaction,[15] and is subsequently hydrolyzed to citrate.

The condensation reaction proceeds by deprotonation of acetyl-CoA by a base on the enzyme, to form the enol or enolate of the substrate, which is the nucleophile for attack on oxaloacetate. Deprotonation of acetyl-CoA is thought to be rate-limiting for the overall reaction.[16] It appears unlikely that this step is concerted with the carbon-carbon bond formation of the condensation reaction, as an intermediate has been detected in the enolization of thioacetyl-CoA by CS,[17] and the related Claisen condensation catalyzed by malate synthase has been shown to follow a stepwise mechanism.[18] Attention has therefore focused on the identity of the nucleophilic intermediate, a question linked to the function of the catalytic residues. Crystallographic studies,[19] supported by site-directed mutagenesis results,[20] have indicated that Asp-375 (the numbering used is for pig CS) is the likely base for deprotonation of acetyl-CoA. His-274 donates a hydrogen bond to the carbonyl oxygen of acetyl-CoA and has been proposed to act as the general acid in the reaction. It was suggested that these two proton transfers occur in a concerted manner, to avoid forming the enolate of acetyl-CoA, which was thought to be too unstable to be an intermediate.[5,19,20] His-320 is observed to hydrogen bond to oxaloacetate, and was proposed to act as a proton donor in the formation of citryl-CoA.[19] Several groups have suggested the involvement of Asp-375 in the hydrolysis of citryl-CoA, but the mechanism of this stage of the reaction remains unclear and poorly characterized. It may be coupled in some way to a conformational change, from the closed to the open form of the enzyme, which allows product release.

It was believed that His-274 must be positively charged to act as a general acid. In the light of simulation[21,22] and NMR[23] results showing that the electrophilic or general acid catalyst His-95 in TIM is neutral, it was pointed out that the hydrogen bonding environment of His-274 in CS indicates that it too is neutral.[24] This suggested that CS and TIM may both utilize neutral imidazole as an acid, or that another mechanism might be operative. His-320, thought to be the general acid in the condensation reaction, also appears to be neutral. Recently it has been proposed that the neutral His-274 side chain stabilizes the enolic intermediate in CS by a very strong, short hydrogen bond.[25]

In the present work, we report QM/MM calculations of the reaction pathway for enolization of acetyl-CoA by citrate synthase. This reaction step is of particular interest as the apparent rate-limiting step and because of its relation to other enzymic carbon acid deprotonations. The aims were to examine the relative stabilities of the different forms of acetyl-CoA within the enzyme, to establish the nature of the nucleophilic intermediate and the means of its stabilization. Analysis of approximate energetic contributions to the reaction allows important interactions within the enzyme to be identified. The results are compared to a Marcus formalism analysis of the reaction and various mechanistic proposals. The QM/MM method has been tested for the problem under consideration, and found to be reliable. The results are consistent with experimental and theoretical investigations, and shed light on the catalytic strategy of CS, with implications for other enzymes. (see Tables I and II.)

METHODS

The combined QM/MM potential[2] implemented in the CHARMM program[26] was applied to model the reaction. In this technique a small region at the active site of an enzyme is treated by a semiempirical molecular orbital (MO) method (in this case AM1[27]), whereas the surrounding protein is included by a simpler MM representation. The QM atoms are influenced by the partial charges of the MM atoms, and bonded and van der Waals interactions between the two regions are included consistently. This method has been used successfully to model the reactions catalyzed by TIM,[21] chorismate mutase,[28] and sialidase,[29] and also reactions in solution,[30–32] solvation processes,[33] and DNA crosslinking by nitrous acid.[34] The calculations were carried out with a modified version of CHARMM version 23.[35] Test calculations on small models of the reaction at the semiempirical MO level with the AM1, PM3, and

MNDO methods were carried out with the MOPAC 6.0 program,[36] and at the RHF/6–31+G(*d*) and MP2/6–31+G(*d*) ad initio MO levels with gaussian-90/92.[37]

Choice of Starting Structure

Important insight into the structure and mechanism of citrate synthase has come from crystallographic investigations, in particular by Remington and coworkers.[8,24,38] Binding of oxaloacetate or other high affinity ligands induces a change in CS conformation from an "open" to a "closed" form, which is observed crystallographically[10] and by other techniques.[39] Informative details of the pathway of this conformational change have come from simulations[40] and structural analysis.[41] It is believed that the closed form catalyzes all the steps in the reaction, as substrates, products, and intermediate analogs have all been observed bound to this form.[19] The function of the open form is product release. There have, however, been suggestions that a conformational change occurs between the condensation and hydrolysis steps.[42,43]

The starting point for the calculations was the 1.9 Å resolution structure of the ternary complex of chicken CS with acetyl-CoA and *R*-malate,[44] (Protein Data Bank[45] code 4CSC.PDB), which has the closed conformation. For meaningful calculations to be performed, it is essential that a high-resolution structure is used, and that this accurately represents the enzyme-substrate complex. *R*-Malate binds to the enzyme in the same conformation as oxaloacetate, whilst the structure of acetyl-CoA and the rest of the active site is appropriate for the reaction, judged by comparison to other CS complexes (for example, with "TS analogs" or high-affinity inhibitors[8,19]). This structure should therefore serve as a good model of the reactive ternary complex. The dimeric enzyme was constructed by symmetry from the monomer structure.

In the closed conformation of CS, the active site is buried and inaccessible to bulk solvent, although some ordered water molecules are observed.[38] To test whether other water molecules, not detected crystallographically, may be present at the active site, the following procedure was used. A preequilibrated 15 Å radius sphere of 464 TIP3P[46] water molecules was superimposed on the structure, and those with an oxygen atom within 2.6 Å of any heavy atom in the structure were deleted. Repeating this operation with the sphere centered sequentially on several different atoms in the structure, with various rotations of the sphere, led to the addition of only seven water molecules within 10 Å of the terminal methyl carbon of acetyl-CoA. None were in positions likely to affect the reaction. Accordingly, only crystallographic waters were included in the calculations.

Several water molecules are apparent at the active site. Their low-temperature factors, conservation between structures and location in the interior of the protein indicate that these water molecules definitely bind as part of the structure,[47] and in some cases may have a functional role. In particular, a cluster of three water molecules (Wat-584, 585, and 586) is located in a position to form hydrogen bonds to the carbonyl oxygen of acetyl-CoA, the C1 carboxylate of *R*-malate (or oxaloacetate) and also within the cluster. In view of the possible importance of these hydrogen bonds, calculations on small models of the active site were carried out with the PM3 semiempirical method,[48] which deals accurately with the geometry of water clusters.[49] A favorable and realistic geometry for the three water molecules in the active site was found, and built into the structure.

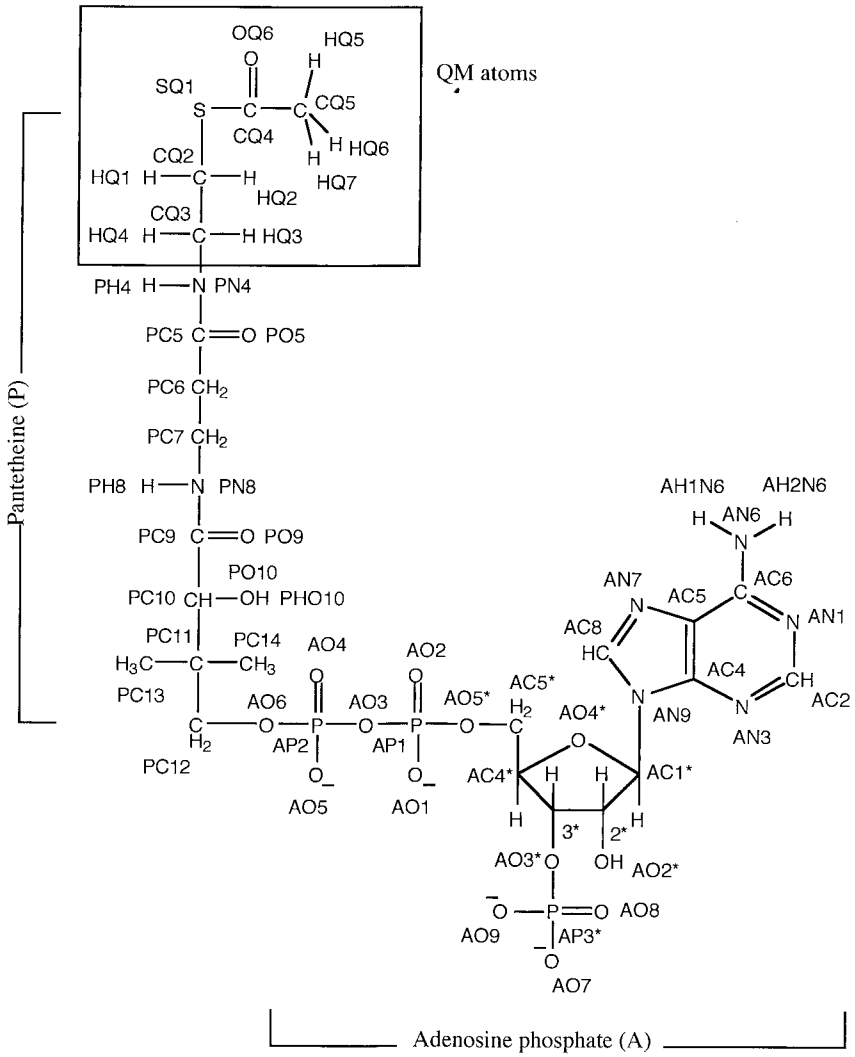
Hydrogens were added to the structure with the HBUILD facility in CHARMM.[50] Minimization of the whole enzyme by purely MM methods was performed to relax any initial strain, and allow the appropriate small changes in geometry as *R*-malate was changed to oxaloacetate. For these minimizations alone, a cutoff of 12 Å was applied to nonbonded interactions; 200 steps of steepest descents minimization were performed for hydrogen atoms only. Following relaxation of two methionines (45 and 127) close to the symmetry axis,[51] oxaloacetate was minimized for 95 steps with the adopted basis Newton-Raphson (ABNR) method to change from the starting structure of *R*-malate; 1000 steps of ABNR minimization were then performed for the water molecules alone, and finally the entire structure was minimized for 392 steps (ABNR). This final structure was used to begin the QM/MM calculations. Some similar MM minimizations were carried out to test the protonation states of active site residues (see below).

Molecular Mechanics Parameters

The CHARMM 22 united atom (polar hydrogens only) parameter set supplied with the QUANTA[52] package was used for the amino acid residues in the MM region. The atoms of acetyl-CoA and oxaloacetate were assigned standard CHARMM/QUANTA atom types appropriate to their chemical environments. Water molecules were represented by the TIP3P model.[46] The modification of adding a van der Waals radius for water hydrogen atoms, commonly made in molecular dynamics simulations, was not applied because this produced erroneous results, as noted previously.[2]

In the QM/MM calculations, oxaloacetate was treated by MM. This substrate binds to citrate synthase as the dianion, and the proximity of this high concentration of negative charge will affect the QM region significantly. The oxaloacetate atomic charges are therefore of particular importance. They were calculated by fitting to the electrostatic potential of the molecule by the CHELPG procedure in the ab initio MO program gaussian-90,[37] at the RHF/6–31G(*d*) level. Very similar charges were found with

TABLE I. Atomic Charges and Atom Types for Acetyl-CoA (Total Charge −4.00), Indicating Division Into Groups†



Group	Atom name	Atom type‡	Charge	Comments
Purine ring				
Total charge 0.00)	AN9	N5R	−0.35	Based on adenine charges and atom types from RNA.RTF
	AC8	C5RE	0.43	
	AN7	N5R	−0.54	
	AC5	CR56	−0.10	
	AC6	C6R	0.77	
	AN6	NP	−0.77	
	AH1N6	H	0.36	
	AH1N6	H	0.36	
	AN1	N6R	−0.77	
	AC2	C6RE	0.79	
	AN3	N6R	−0.73	
	AC4	CR56	0.55	
Ribose ring				
(total charge 0.00)	AC5*	CH2E	0.00	Based on ribose charges and atom types from RNA.RTF
	AC4*	CH1E	0.10	
	AO4*	OE	−0.36	
	AC3*	CH1E	0.06	
	AC2*	CH1E	0.25	
	AO2*	OT	−0.65	
	AHO2*	HO	0.40	
	AC1*	CH1E	0.20	
3′-Phosphate				Based on scaled 3–21+G(d) calculations on GDP (Worth,
(total charge −2.00)	AO3*	OS	−0.461	1992) and DNA.RTF
	AP3*	PO4 Tc1.20		
	AO7	OC	−0.913	
	AO8	OC	−0.913	
	AO9	OC	−0.913	

(Continued)

TABLE I. Atomic Charges and Atom Types for Acetyl-CoA (Total Charge −4.00), Indicating Division Into Groups† *(Continued)*

Group	Atom name	Atom type‡	Charge	Comments
Purine ring				
5′-Diphosphate link				
(total charge −2.00)	AO6	OS	−0.46	Based on scaled 3–21+G(d) calculations on GDP (Worth, 1992) and DNA.RTF
	AP2	PO4	1.20	
	AO4	OC	−0.72	
	AO5	OS	−0.72	
	AO3	OS	−0.60	
	AP1	PO4	1.20	
	AO2	OC	−0.72	
	AO1	OS	−0.72	
	AO5*	OC	−0.46	
-CH_2-$C(CH_3)_2$-				
(total charge 0.00)	PC11	CT	0.00	Based on valine and leucine side chains in AMINO.RTF
	PC12	CH2E	0.00	
	PC13	CH3E	0.00	
	PC14	CH3E	0.00	
-CH(OH)-				
(total charge 0.00)	PC10	CH1E	0.25	Based on serine side chain in AMINO.RTF
	PO10	OT	−0.65	
	PHO10	HO	0.40	
-CH_2CH_2NHCO-				
(total charge 0.00)	PC6	CH2E	0.00	Peptide charges from Nakagawa et al., 1993
	PC7	CH2E	0.20	
	PN8	NP	−0.50	
	PH8	H	0.30	
	PC9	C	0.55	
	PO9	O	−0.55	
-CONH-				Adjacent to QM region; nitrogen charge lowered to make NH unit neutral
(total charge 0.00)	PN4	NP	−0.30	
	PH4	H	0.30	
	PC5	C	0.55	
	PO5	O	−0.55	
QM region				
(atomic charges all zero in QM/MM calculations)	CQ5	CT	$(-0.013)^{\Sigma}$	
	HQ5	HA	$(0.031)^{\Sigma}$	
	HQ6	HA	$(0.031)^{\Sigma}$	
	HQ7	HA	$(0.031)^{\Sigma}$	
	CQ4	C	$(0.55)^{\Sigma}$	
	OQ6	O	$(-0.55)^{\Sigma}$	
	SQ1	SE	$(-0.08)^{\Sigma}$	
	CQ3	CT	$(0.00)^{\Sigma}$	
	HQ4	HA	$(0.00)^{\Sigma}$	
	HQ3	HA	$(0.00)^{\Sigma}$	
	CQ2	CT	$(0.00)^{\Sigma}$	
	HQ1	HA	$(0.00)^{\Sigma}$	
	HQ2	HA	$(0.00)^{\Sigma}$	

*AMINO.RTF, DNA.RTF, and RNA.RTF refer to residue topology files developed for the QUANTA/CHARMM programs by Molecular Simulations, Inc.

†Figure: Acetyl-CoA, indicating QM/MM partitioning and atom naming. The atoms of the MM part of the molecule are named according to the scheme used for the entries in the Brookhaven National Laboratory Protein Data Bank.

‡Standard QUANTA/CHARMM atom types (QUANTA: Parameter Handbook, Version 3.3, Molecular Simulations Inc. 200 Fifth Avenue, Waltham, MA 02154).

$^{\Sigma}$MM charges are only used for these atoms in initial minimizations treating the whole enzyme by MM. In the QM/MM calculations, these atoms are treated by AM1, and assigned MM charges of zero.

TABLE II. Atom Types and Atomic Charges for Oxaloacetate (Total charge −2.00)*

Atom number	Atom type†	Charge (6–31G(*d*))
C1	C	0.92
O1	OC	−0.88
O2	OC	−0.88
C2(+H1+H2)‡	CH2E	−0.18
C3	C	0.51
O7	OK	−0.61
C6	C	0.78
O5	OC	−0.83
O6	OC	−0.83

*Charges derived by a least-squares fit to the RHF/6–31G(*d*) electrostatic potential.

†Standard QUANTA/CHARMM atom types (QUANTA: Parameter Handbook, Version 3.3, Molecular Simulations Inc. 200 Fifth Avenue, Waltham, MA 02154).

‡Treated as a united atom, including H1 and H2.

higher level (MP2/6–31+G(*d*)) calculations. Different charge sets for oxaloacetate were used in test calculations, and did not significantly alter the results.

Atomic partial charges for the groups in acetyl-CoA were assigned based on QUANTA/CHARMM parameters and ab initio MO calculations for similar molecules.[53,54] A full list of the oxaloacetate and acetyl-CoA atomic charges is available as supplementary material from the authors.

QM/MM Calculations

In the QM/MM calculations, two different sizes of system were studied, based on spheres of radius 17 Å and 20 Å, respectively, around the terminal methyl carbon of acetyl-CoA in one active site. It was found that smaller systems than these produced unrealis-

tic distortions of the structure because not all the acetyl-CoA binding interactions were included. In each case, all residues with at least one heavy atom within the selection radius were included, and the remaining atoms deleted. Both simulation systems contained the whole of acetyl-CoA, and all the residues involved in binding this substrate. The boundaries of the simulation systems were restrained with conditions similar to the stochastic boundary method of molecular dynamics.[55] Each system was divided into three zones based on the starting structure. The central or "reaction" zone consisted of all atoms within 14 Å of the terminal methyl carbon, and was subject to no restraints. The "buffer" zone included all atoms lying at distances between 14 Å and 16 Å away. Harmonic restraints were applied to atoms in the buffer zone, scaled from a maximum at 16 Å to zero at a distance of 14 Å from the center. Force constants for different atom types were calculated from approximate average x-ray temperature factors.[55] The outer "reservoir" zone contained all atoms further than 16 Å from the center. The same boundary conditions were applied in the 17 Å and 20 Å radius systems, which differed only in the size of the reservoir zone and hence the number of fixed atoms. The 17 Å radius system contained 1717 atoms in total, whereas the 20 Å radius system contained 2582.

Partitioning a system into QM and MM regions must be done with care, to represent the active site accurately. "Link" atoms (AM1 hydrogen atoms) fulfill the valence requirements for QM atoms where the boundary between the QM and MM regions separates covalently bonded atoms.[2] After testing various alternatives, the QM region adopted for the calculations contained the side chains of Asp-375 and His-274, up to and including Cβ, and the thioester portion of acetyl-CoA (equivalent to ethylthioacetate) for a total of 33 QM atoms, including three link atoms. The total charge of the QM region was −1 a.u. For Asp-375 and His-274, both Hα and Cα (which are adjacent to the QM region) were represented explicitly, rather than by a single united atom, and their charges were set equal and opposite to avoid unrealistic polarization.

No cutoff was applied for the nonbonded interactions in the QM/MM calculations, and a constant dielectric of unity was used. It should be noted that solvent dielectric screening effects are not directly included in this model. The substrate complex was minimized by steepest descents (250 steps) and then by the Powell conjugate gradient method until the root mean square (RMS) gradient fell below 0.01 kcal/Å (approximately 1000 steps). This convergence criterion was employed throughout.

Reaction Path Generation, Testing, and Analysis

Energy profiles for the reactions in citrate synthase were calculated by an adiabatic mapping procedure, minimizing the total energy along an accurate reaction coordinate for each of the transformations. For a full treatment of a chemical reaction, whether in the gas or a condensed phase, the dynamics of the process should be included.[56–58] However, studies of the potential energy surfaces governing enzymic reactions can in themselves be mechanistically revealing,[21,22,28,59] and are an essential precursor to more extensive dynamic studies. In the present case, details of the energy profile, especially the relative stabilities of the various forms of acetyl-CoA, and the associated potential barriers, may be expected to play a central role in governing reactivity. The adiabatic mapping approach is reasonable for the simple proton transfers under consideration here, which are unlikely to be accompanied by major conformational changes, and given that the structure is a good model of the reactive complex.

It is essential that a good reaction coordinate is used in order to represent the reaction properly. Calculating reaction paths for chemical or conformational changes in enzymes is a challenging problem because of the large size of the systems.[1] This presents difficulties both computationally, in characterizing true TSs (saddle points) and their associated pathways, and because of the complex nature of protein potential energy surfaces.[60,61] To ensure that the reactions studied here were represented accurately, the applied reaction coordinates were based on exact AM1 TSs and minimum energy pathways obtained for small models.[62]

AM1, in common with other semiempirical MO methods, has acknowledged weaknesses, for example in dealing with TSs in some cases.[63] Its accuracy in the present case has been established by comparison with high-level ad initio calculations on related molecules, and small model complexes and TSs representing the reaction.[21,64] As an alternative to AM1, another MNDO-type method, such as PM3, could have been used in the QM/MM calculations. AM1 and PM3 deal better with hydrogen bonded systems than does MNDO itself, but debate continues over the relative merits of these methods.[36,49,63,65] For many applications, PM3 performs better than its predecessor, AM1. However, due to an apparent error in its parameterization,[66] PM3 calculates charges for nitrogen atoms in N-H groups which are erroneously positive, leading to an incorrect description of the electrostatic potential of histidine. PM3 was also found to give poorer results for the enolate-enol reaction than AM1, compared to ab initio results. Overall AM1 was found to perform satisfactorily for the whole reaction, and was therefore used in the QM/MM calculations. Model calculations of hydrogen bond energies were repeated with PM3, for comparison with the AM1 results.

To calculate the energy profile in the QM/MM calculations for a given reaction step, a harmonic restraint was applied to hold the chosen reaction

coordinate close to its value in the model TS. After experimenting with a variety of reaction coordinates (for example, various interatomic distances), it was found that a good reaction coordinate to describe proton transfer between atoms A and B is the ratio of the A-H distance to the A-B distance. The force constant for restraining this reaction coordinate (ratio of interatomic distances) was 10,000 kcal/mol, a large value designed to keep the system close to the desired reaction pathway.[21] The approximate TSs were modeled by minimizing the whole system for a maximum of 1000 steps of steepest descents (SD), or until the gradient converged. The products of each step were then minimized with no restraints applied to the active site (up to 500 steps of SD and 1000 steps of Powell conjugate gradients). The fully minimized enolate structure was used as the starting point for the enolate-enol reaction. In the 17 Å radius system, further intermediate points along the reaction profiles between reactants and products were studied by varying the value of the reaction coordinate, and performing up to 250 steps of SD at each point. To test the results, the reaction was simulated in reverse also by the same method, beginning with the final fully minimized enol structure, and proceeding via the enolate to the keto (substrate) form of acetyl-CoA. The energy profiles for the forward and reverse reactions were virtually identical.

An approximate analysis of the energetic effects of individual residues on the reaction was performed by deleting residues in order of their center of mass distance from the active site (in the starting structure), furthest first, and recalculating the energy differences between reactants and products for each step. This method has been applied previously to estimate residue contributions to the TIM reaction.[21] This analysis should indicate qualitatively which residues significantly affect the reaction, although it should be borne in mind that the resulting energies are simply differences in interaction energies for the structures, and not free energies, which could, for example, be obtained from mutation experiments. Solvent dielectric screening was not directly included, and will affect the interactions, particularly for groups close to the protein surface, but this should not markedly alter the results within the deeply buried CS active site.

RESULTS

Protonation States of Active Site Histidines

The imidazole side chain of histidine is found in proteins in two neutral tautomeric forms (protonated on Nδ1 or Nϵ2 alone), and in the positively charged (doubly protonated) imidazolium form. The typical pK_a of histidine in small peptides is approximately 6.5, but this can be significantly altered by environmental effects in a protein.[23] It is often difficult to assign the correct protonation states to histidine residues in crystal structures.[67] Two conserved histidine residues in citrate synthase (His-274 and His-320 in pig CS) are believed to be catalytic. Their protonation states are of great importance to the mechanism, and were therefore investigated. Both were believed to be positively charged in the active form, although more recently it has been suggested that they are in fact neutral.[24]

Calculation of the pK_as of ionizable side chains in proteins is a topic attracting much interest in biophysics.[68–70] Techniques based on solution of the Poisson-Boltzmann equation by finite difference methods show great promise, but do suffer from some uncertainty.[71] For histidine side chains, examination of the hydrogen bonding environment can provide a good guide to protonation state.[67] Energy minimization was used here as an extension of this simple means of addressing the favored protonation states of active site histidine residues. The starting structure of the CS dimer was energy minimized by purely MM techniques, with His-274 and His-320 either positively charged or neutral (His-274 protonated singly on Nδ1, His-320 on Nϵ2, as indicated by hydrogen bonding). With these residues treated as positively charged, energy minimization caused a reorientation away from their positions in the crystal structure, and the active site became clearly disrupted. His-274 rotated away from acetyl-CoA, breaking the hydrogen bond with the carbonyl oxygen, to a position where it could no longer easily transfer a proton to the substrate. Its position at the N terminus of helix L changed radically, and the interaction with Ser-244 (see below) was not maintained. A similar finding was noted in studies on TIM, indicating the neutrality of His-95.[21,22] The hydrogen bond between His-320 and oxaloacetate was also lost. In contrast, with both histidines neutral the structure of the active site remained close to the starting structure, and the hydrogen bonds involving these residues were maintained. The same effects were apparent with different minimization conditions and parameter sets. Therefore it appears that His-274 and His-320 are indeed neutral in the crystal structure, and they were treated as such in the QM/MM calculations. Preliminary Poisson-Boltzmann calculations with the DelPhi[72] program supported the conclusion that the neutral forms of these residues are favored at the active site.

Two other histidines (His-235 and His-238 in pig CS) appear to have important structural or functional roles. His-238 is conserved (along with its flanking residues Asp-237 and Glu-238) according to alignments of CS sequences.[73,74] Another histidine is found three places earlier in many CS sequences, indicating that His-235 may also be conserved, although not absolutely. Both residues are close to the reactive system, and could have a significant influence on it.

QM/MM minimization of the 20 Å radius system caused deformation of the active site when His-235

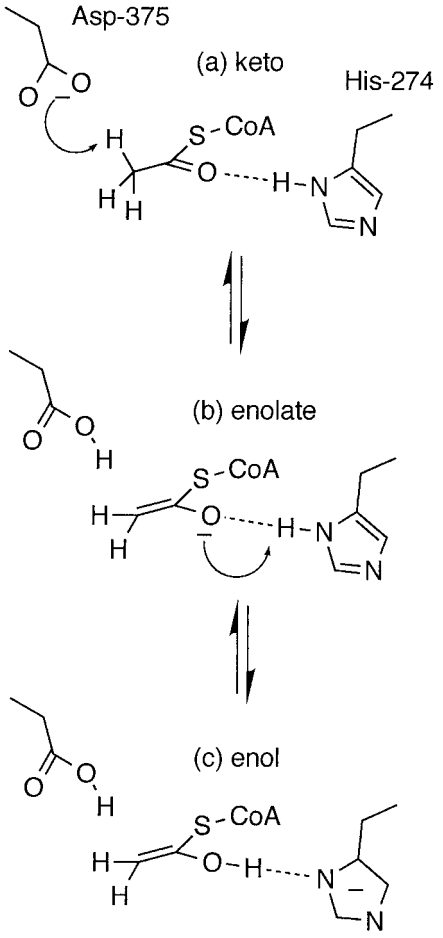


Fig. 2. Mechanism of acetyl-CoA enolization by citrate synthase, indicating the keto, enolate, and enol forms of acetyl-CoA.

was positively charged. The carboxylate side chain of Asp-375 shifted and rotated to a position where it was no longer favorably oriented to abstract a proton from acetyl-CoA. With His-235 treated as neutral (Nδ1 tautomer), Asp-375 remained in its crystallographic position. Repeated minimizations with slightly different starting coordinates and conditions gave similar results. The neutral form was therefore used for His-235 in the calculations on the reaction. This residue appears to have a structural function, maintaining the orientation of the catalytic Asp-375 through an interaction with a bridging water molecule (Wat-596). His-235 is itself restrained by a hydrogen bond with the backbone and aromatic interactions.

His-238 donates a hydrogen bond from Nδ1 to a carboxylate group of oxaloacetate, and appears to 'stack' against the guanidinium group of Arg-329, which is also involved in binding this substrate. QM/MM minimizations gave similar results with His-238 treated as neutral (Nδ1) or positively charged. Nϵ2 of the imidazole side chain protrudes into a cavity in the protein where it may interact with a water molecule as a hydrogen bond donor or acceptor. The protonation state of His-238 was therefore not clear from these considerations. However, one small but notable difference was that with His-238 neutral, but not when it was positively charged, the hydrogen bond between a water molecule (Wat-585) and the carbonyl oxygen of acetyl-CoA was broken by energy minimization because of reorientation of the water. This effect was found in several calculations under different conditions. The hydrogen bond had been inferred from the crystal structure,[44] and subject to errors in the potential provides a slight indication that it may be correct to treat His-238 as positively charged. In the 17 Å radius system, the positively charged form was used for His-238. Separate calculations on the 20 Å radius system were performed with this residue neutral or positively charged, to test the effect of the charge on the reaction.

Energy Profiles

Figure 3 shows the variation in the total energy of the system along the reaction pathway calculated as described above. The stages of the reaction are denoted by the acetyl-CoA species involved, namely, the keto (substrate/reactant), enolate, and enol forms (Fig. 2). The left-hand side of the profiles is for the conversion of the keto to the enolate form, by transfer of a proton from the acetyl methyl carbon to a carboxylate oxygen of Asp-375. For this step, the applied reaction coordinate was the ratio of the O-H to C-O distances for the reacting atoms. The right-hand side of the profile refers to the enolate to enol reaction, in which a proton is transferred from Nδ1 of His-274 (forming the imidazolate), to the enolate oxygen of the substrate. The reaction coordinate for this step was the ratio of the O-H and N-O distances. Calculations on small models[64] indicate that with His-274 neutral, concerted formation of the enol from the keto form, without formation of the enolate, does not occur. No evidence for the concerted reaction in the enzyme was found in the QM/MM calculations either. The calculations reported here therefore only deal with the stepwise keto-enolate-enol reaction.

The energy profile calculated for the 17 Å radius system (His-238 positively charged) is shown in Figure 3i,ii shows the results for the 20 Å radius system. Essentially identical plots were obtained from calculations on the reverse reactions. The results for the 17 Å and 20 Å simulation systems are also similar. The energy maximum along the (keto to enolate) reaction path in the 17 Å radius system is only slightly different from the AM1 TS taken as a model for the reaction, and that for the enolate to enol reaction is almost exactly the same as the TS for

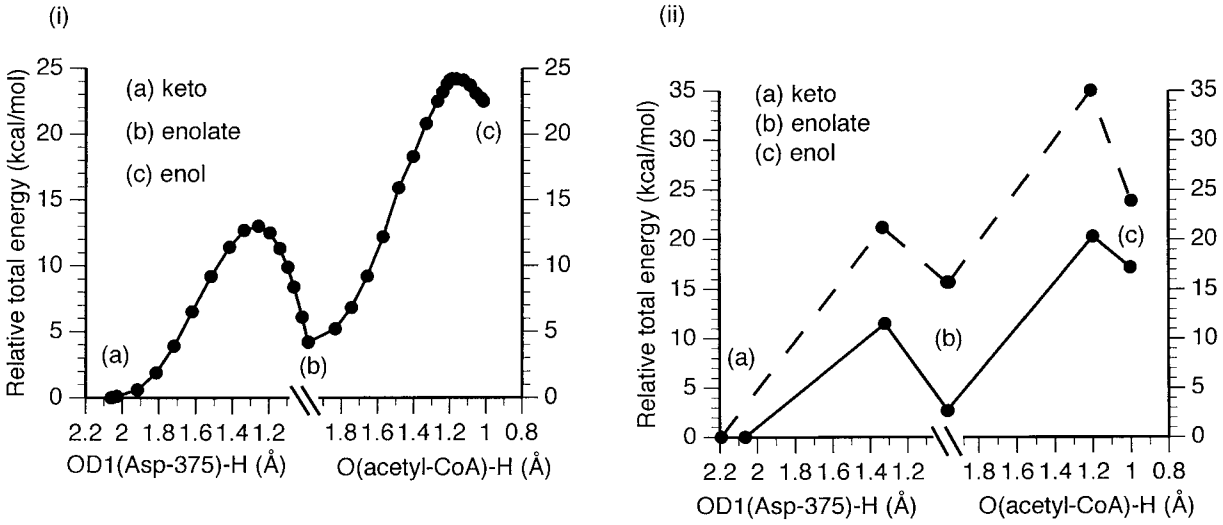


Fig. 3. **(i):** Energy profile for the conversion of the keto form of acetyl-CoA (a) to the enolate (b), and enolate (b) to enol (c) in citrate synthase, in the 17 Å radius simulation system (His-238 positively charged). **ii:** Energy profile for the reaction in the 20 Å radius system. The dotted line shows the results with His-238 treated as neutral, the solid line the results with His-238 positively charged.

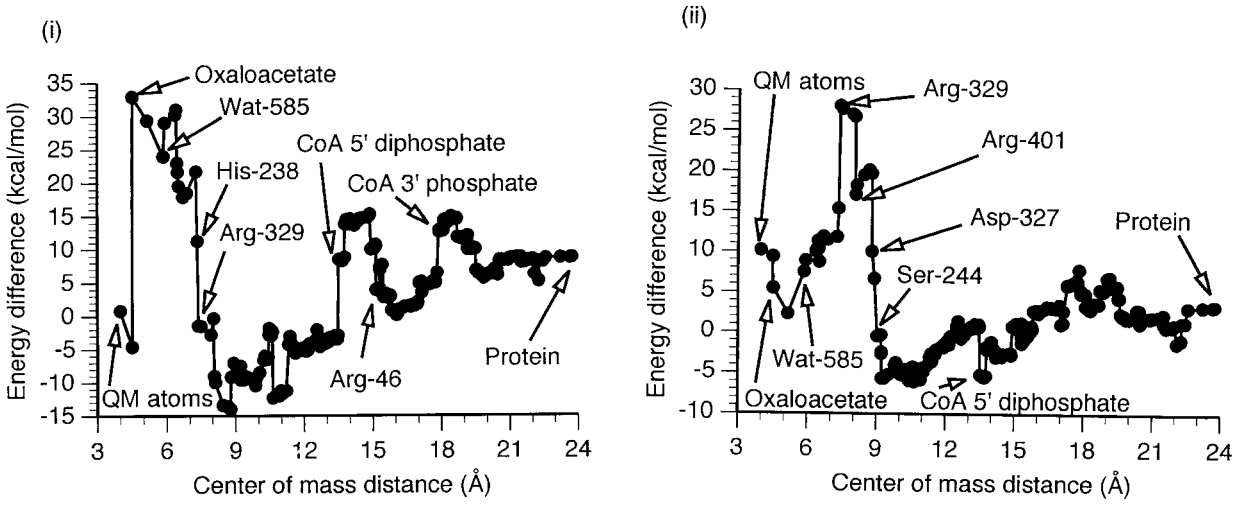


Fig. 4. **i:** Contributions of MM groups to the QM/MM energy difference between the keto and enolate forms (*E*(enolate)—*E*(keto)). **ii:** Residue contributions to energy difference between the enolate and enol forms (*E*(enol)—*E*(enolate)). The energy differences are plotted against the center of mass distance of the MM residues from the center of the 20 Å radius simulation system. Some important contributions are indicated. "Protein" indicates the energy difference found for the whole simulation system, and "QM atoms" refers to that for atoms in the QM region only, with all other atoms deleted.

this step. This confirms that the gas phase results are good structural models for the reaction in the enzyme. The relative energies of the three forms are significantly affected by the charge of His-238, as can be seen from the energy profiles for the 20 Å radius system (Fig. 3ii). In particular, this residue stabilizes the enolate form when treated as positively charged. However, the stability order of the three forms is not changed. The magnitude of this interaction may also be reduced by dielectric screening.

Significant barriers are found for both the keto to enolate and enolate to enol reactions. In all cases the keto form of acetyl-CoA is calculated to be most stable, and the enolate is more stable than the enol. The structure of the active site is largely unchanged by the proton transfers, with only slight shifts of position observed for the catalytic residues. A notable structural feature is the shorter hydrogen bond with His-274 in the enolate (N-O distance approximately 2.96 Å) and enol (N-O distance 2.74–2.85 Å)

than in the keto form (N-O = 3.08 Å). This indicates a strengthening of the acetyl-CoA-His-274 hydrogen bond when one of the partners is charged (the enolate of acetyl-CoA, or the imidazolate of His-274 in the enol complex). The hydrogen bond is an important factor in stabilizing the intermediate relative to the keto form, as discussed below.

Residue Contributions

The effects of individual MM groups on the relative energies of the keto, enolate, and enol complexes were estimated by deleting them sequentially in order of distance from the active site, and recalculating the energies. The resulting (enolate-keto) and (enol-enolate) energy differences plotted versus the center of mass distance of the deleted residues are shown in Figure 4i,ii. Some groups making large contributions are indicated. This method is clearly approximate, and the resulting interaction energies should be interpreted simply as a guide to which residues and groups (in particular those in close proximity to the active site) significantly affect the reaction energetics. The calculations were performed on the 20 Å radius system with His-238 positively charged. As has been found for other enzymes,[21,75] electrostatic interactions are seen to play a crucial part in determining the reaction energies, and their size and long-range stress the importance of including the enzyme environment in accurate calculations. It should, however, be remembered that all electrostatic interactions within the protein, especially for groups close to the surface, will be affected by solvent dielectric shielding. For example, experimental measurements of the effect of varying surface charge on the binding of charged ligands to the active site of carbonic anhydrase II demonstrate the attenuation of electrostatic interactions in solution.[76]

The effects of charged groups can be understood from their proximity to the concentration of negative charge in the QM region. Thus in the keto complex, the negative charge is concentrated on Asp-375, in the enolate on acetyl-CoA, and in the enol complex on His-274. The effects of even relatively distant charged groups can be significant, although these will be overestimated in the absence of solvent. The individual interaction energies are large, and it is important to consider the overall net effect. For example, the destabilization of the enolate relative to the keto form by oxaloacetate is counterbalanced by positively charged binding residues.

Some nearby neutral groups also make important energetic contributions. For example, a water molecule (Wat-585) which donates a hydrogen bond to the carbonyl oxygen of acetyl-CoA, stabilizes the enolate form relative to both the keto and enol forms (by 5.4 and 5.2 kcal/mol, respectively). This water molecule is part of the ordered, conserved cluster of three water molecules bound between oxaloacetate and acetyl-CoA, as mentioned above. Wat-596, on the other hand, hydrogen bonds to Oδ2 of Asp-375, and stabilizes the keto complex by a similar amount.

Val-243 and Ser-244 lie at the N-terminus of helix L, close to His-274. The positions of these residues suggest that they could donate hydrogen bonds to Nϵ2 of His-274, enforcing its neutrality and lowering its pK_a.[24] Both are calculated to stabilize the enol complex relative to the enolate, although neither backbone NH group makes a good hydrogen bond with His-274. Val-243 is calculated to have only a small effect, but the effect of Ser-244 is large and notable (stabilization of the enol by 7 kcal/mol relative to the enolate, and by 9 kcal/mol relative to the keto complex). In the minimized structures, which are very similar to the crystallographic structures, the hydroxyl side chain of Ser-244 (a conserved residue) donates a hydrogen bond to Nϵ2 of His-274. His-274 in turn hydrogen bonds through Nδ1 to the carbonyl (or enolate) oxygen of acetyl-CoA. This arrangement, which has not previously been noted, is reminiscent of the catalytic triad of Ser-His-Asp found in serine proteases and other enzymes. A Cϵ1-H···O interaction between His-274 and oxaloacetate, of the type observed for other catalytic triads and thought to be functionally important,[77] is also apparent. It is interesting to note that the serine protease catalytic triad involves charged hydrogen bonding in the ground state.[78–80] Ser-244 serves to position and ensure the neutrality of His-274, suggesting a reason for its conservation. It may also modulate the interaction of the imidazole with acetyl-CoA somewhat, through cooperative effects.

DISCUSSION

The fast rates at which certain enzymes, such as citrate synthase, deprotonate carbon atoms adjacent to carbonyl groups are puzzling because they appear to be incompatible with the instability of postulated reaction intermediates. The observed rate for pig CS (k_{cat} = 96 s^{-1} [20]) gives an apparent activation energy, $\Delta G‡$, of 14.7 kcal/mol, an upper limit for the activation energy of any step in the reaction.[79] From the pK_as of Asp-375 (6.5 [81]) and acetyl-CoA (approximately 20.4–21.5, based on results for a thioester[82]) the products of the proton abstraction reaction may be estimated to be approximately 20 kcal/mol higher in energy than the reactants ($\Delta G°$ = 20 kcal/mol). This free-energy difference must be reduced by the enzyme if the enolate is to participate in the reaction.

As a solution to this apparent dilemma, the enol of acetyl-CoA has been proposed as the intermediate, formed in a concerted reaction with Asp-375 as the base and His-274 as the acid.[19,20] To serve this function, it was suggested that His-274 is positively charged, but the results here support the apparent

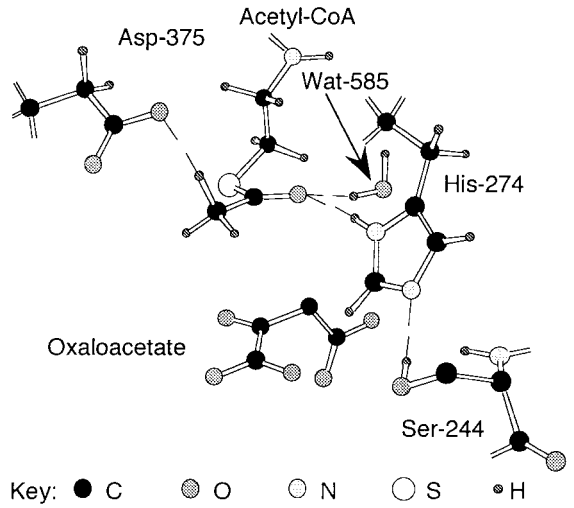


Fig. 5. Detail of the active site of the citrate synthase/substrates complex after QM/MM minimization. Some important interactions are shown by dashed lines.

neutrality[24] of this residue. To form the enol, the neutral imidazole must lose a proton, giving the negatively charged imidazolate. This has a possible* precedent in the TIM reaction, but would mean that the enol is not significantly more stable than the enolate within the enzyme. From the pK_a of His-274 (estimated at 11–14[83]) and the pK_E for the keto-enol equilibrium (10–11 by analogy with ketones,[84]), $\Delta G°$ for enol formation can be estimated as $\Delta G° \geq\sim 20$ kcal/mol in the absence of stabilizing factors.

The simulations support the identification of Asp-375 as the base for deprotonation of acetyl-CoA by CS. This residue is well positioned to perform this function, as the TS for proton transfer can be reached easily without structural rearrangement. The barrier to reaction is also reasonable. An alternative proposal, that His-274 is the base in the reaction[43,85] is unlikely; this would require considerable change of the active site structure, including the reorientation and breaking of hydrogen bonds of His-274. There are no other obvious candidates for the catalytic general base.

In all the calculations the enolate form of acetyl-CoA was found to be more stable than the enol. This confirms that within the enzyme the enol offers no advantage in stability over the enolate. No evidence was found for a concerted mechanism. As expected, the keto (substrate) is the lowest energy form. The energies relative to the keto form indicate that the enolate can be an intermediate in the reaction. However, the calculated energy differences depend strongly on the protonation state of His-238. In its positively charged form, this residue stabilizes the enolate. With His-238 treated as positively charged, the enolate-keto energy difference is relatively small (2.7 kcal/mol and 4.2 kcal/mol in the 17 Å and 20 Å radius systems, respectively), whereas treating His-238 as neutral (20 Å radius system) the enolate-keto energy difference is calculated to be considerably larger (15.7 kcal/mol). Although solvent dielectric screening will affect electrostatic interactions within the enzyme and reduce this effect, this again emphasizes their importance.

Large barriers were found for both the keto to enolate and enolate to enol reactions. Modeling proton transfers with TS structures has some shortcomings because of the neglect of tunneling and other effects, but the adiabatic barrier calculated in this way can provide a reasonable first approximation. The barriers for enolate formation from the keto complex are consistent with this proton abstraction being rate-limiting.[16] A barrier is also found for the reverse (enolate to keto) reaction, further evidence that the enolate can have a finite lifetime in the active site of CS. The barrier to the enolate to enol reaction is large in all cases, and although there are indications that this may be overestimated by the AM1 method,[64] this is clearly a significant hurdle to enol formation.

The Role of Hydrogen Bonds in Catalysis

During the course of this work, an explanation was put forward for the fact that the general acid residues in many enzyme-catalyzed enolizations are neutral, despite mechanistic expectations.[25] It was suggested that a hydrogen bond between an enolic intermediate and a neutral acid at the active site could be very strong, and lower the activation energy by stabilization of the enolic intermediate and entropic ("solvation") effects. The term "enolic intermediate" was used to denote the fact that in a strongly hydrogen bonded system the barrier to proton transfer may be very low, and the proton may effectively be shared between the bonded partners. The intermediate is then neither the enolate nor enol in isolation, but lies somewhere between these two extremes. It has been proposed that "short, strong" or "low-barrier" hydrogen bonds, which can be exceptionally strong in the gas phase, stabilize intermediates in a wide variety of enzymic reactions.[6,7] Low-barrier hydrogen bonds were proposed to form between partners of approximately equal pK_a, when one is charged and if bulk solvent is excluded. They have been proposed to be responsible for low fractionation factors found for some enzymic bases.[86] Unusually short hydrogen bonds in enzymes have been observed in crystallographic investigations of citrate synthase complexes with inhibitors.[8] Ultraviolet spec-

*Recent ab initio calculations by Kollman and coworkers[22] show no tendency for His-95 to donate a proton to the enediolate intermediate in TIM, in full agreement with the results presented here. These workers propose that the TIM mechanism instead proceeds by an intramolecular proton transfer.

troscopic shifts[87] and NMR[88] results have been interpreted as evidence for low-barrier hydrogen bonds, although alternative assignments have been made.[78] Such bonds have been proposed to supply 10 to 20 kcal/mol to stabilize intermediates in enzymic reactions.[7] Experimental measurements[8] indicate, however, that a very short hydrogen bond with an inhibitor at the active site of citrate synthase provides only a little more stabilization than an analogous slightly longer bond in a related inhibitor complex (Fig. 5). Theoretical studies[89] suggest that formation of low-barrier hydrogen bonds will not occur in enzymic catalysis, as the polar environment of the active site will favor an asymmetric double-well potential. Ab initio calculations show no special stabilization associated with pK_a equalization or the disappearance of the proton transfer barrier in hydrogen-bonded systems.[90]

Analyses in terms of Marcus formalism have proved a useful tool in the interpretation of reaction energetics, and for estimating the stabilization of intermediates in enzymes.[25,91,92] This formalism allows the overall activation energy, $\Delta G^{\ddagger}$, for the first-order reaction of enzyme-bound substrates to be related to the free energy difference, $\Delta G°$, between the reactants and products of the reaction step, and an "intrinsic" activation energy, $\Delta G^{\ddagger}_{int}$, characteristic of the reaction type [Eq. (1)]:

$$\Delta G^{\ddagger} = \Delta G^{\ddagger}_{int}(1 + \Delta G°/4\,\Delta G^{\ddagger}_{int})^2 \qquad (1)$$

Gerlt and Gassman[25] proposed that a strong hydrogen bond formed between an enolic intermediate and a neutral acid at the active site could lower both the $\Delta G°$ and $\Delta G^{\ddagger}_{int}$ components of the barrier, and explain the rates of enzymic carbon acid deprotonations. Achieving rapid catalysis of such reactions faces two problems. First, the (enolate or enol) products of the deprotonation are highly unstable with respect to the reactants (keto form), that is, $\Delta G°$ is large. Second, the intrinsic barrier for carbon acid deprotonation in solution is also large ($\Delta G^{\ddagger}_{int}$ = 10–13 kcal/mol[25]). In common with other findings indicating that a lower reorganization energy (compared to solution) in an ordered active site is an important factor in enzymic catalysis,[3,75,92,93] this proposal suggested a reduction in $\Delta G^{\ddagger}_{int}$ by specific solvation of developing negative charge in the reaction by the preformed hydrogen bond, lessening the entropic penalty of solvent rearrangement during the reaction. A small reduction in the intrinsic activation energy ($\Delta G^{\ddagger}_{int}$) in this way would then require only a modest stabilization of the intermediate ($\Delta\Delta G° \sim 7$ kcal/mol) by the enzyme, via a strong hydrogen bond between the intermediate and the general acid.

Several lines of evidence indicate that this analysis requires modification. For example, it now appears that the reduction in $\Delta G^{\ddagger}_{int}$ provided by the active site hydrogen bond is less than that proposed by Gerlt and Gassman. Ab initio calculations show a large barrier to carbon acid deprotonation in the gas phase, even in the absence of a thermodynamic barrier, suggesting that the intrinsic barrier is due mostly to electronic rather than solvation effects, unless proton tunneling is significant.[94] Similarly, calculations on the CS mechanism reported here, and studies on small models,[64] show large potential barriers to proton abstraction from acetyl-CoA. If a large kinetic barrier of this type must be overcome for the reaction within the enzyme ($\Delta G^{\ddagger}_{int}$ is large), then in terms of Marcus formalism $\Delta G°$ must be reduced still further to produce an activation energy equal to or less than the experimentally deduced value. Experiments suggest that this situation pertains to at least one enzyme that deprotonates a carbon acid: evaluation of the internal equilibrium constant in 3-oxo-Δ^5-steroid isomerase[95] showed that the substrate and intermediate are of similar energy when bound to the enzyme ($\Delta G°$ is reduced by 10 kcal/mol from its solution value to less than 1 kcal/mol, whereas $\Delta G^{\ddagger}_{int}$ is reduced only slightly, from 13 to 10 kcal/mol).

Based on these findings, Marcus formalism analysis of the citrate synthase enolization reaction, with $\Delta G^{\ddagger}_{int}$ = 10–12 kcal/mol, indicates that $\Delta G°$ must be reduced by 11.5–15 kcal/mol by the enzyme to be consistent with the experimental result.[64] For some enzymes, such as mandelate racemase[91] and malate synthase, a metal ion may supply at least some of the necessary stabilization of the intermediate electrostatically. CS, however, does not employ a metal ion in catalysis, and the stabilization of the intermediate relative to substrates must be achieved in other ways.

According to the short, strong or low-barrier hydrogen bond hypothesis, the hydrogen bond between His-274 and acetyl-CoA becomes very strong on removal of a proton from the substrate and stabilizes the intermediate. His-274 and the acetyl-CoA enol are proposed to have similar pK_as, and share the proton in a low-barrier hydrogen bond. The present work indicates that the increased strength of this hydrogen bond on enolate formation does indeed make a central contribution toward stabilizing the intermediate, but that this hydrogen bond is not of the low-barrier type, as the basicities of the enolate and the imidazole side chain of this residue are calculated to be clearly different. The proton is therefore not "shared" more than in a normal hydrogen bond, and the intermediate is best described as the enolate, rather than an enolic intermediate.

Charged Hydrogen Bonds Stabilize the Enolate

To have high energies, hydrogen bonds do not have to be of the low-barrier type, although the very

strongest interactions of this type show very low or no barriers to proton transfer. A more important general condition is that they are charged. Charged hydrogen bonds are generally found to be very much stronger (in the gas phase) than their neutral counterparts.[96] Hydrogen bond strength increases as the pK_a difference between the bonded partners decreases, but even when pK_as are not exactly matched, charged hydrogen bonds are of considerably higher energy than equivalent neutral bonds. Ab initio calculations confirm that charge, as opposed to equalization of pK_a, is the vital factor in determining hydrogen bond energy in most cases.[90] This conclusion is in agreement with work that showed only a small variation in hydrogen bond strength with bond length and pK_a differences for inhibitors bound to CS.[8] It is apparent that the pK_as of the two components do not have to be equal in order for a strong hydrogen bond to form. Of course, it must be remembered that the properties of hydrogen bonds depend strongly on their environment. Bonds which are very strong in the gas phase can be much weaker in aqueous solution, due to dielectric and dynamic or entropic effects. Charged hydrogen bonds in proteins are in general stronger than those between neutral species, but when exposed to solvent the stabilization may be reduced. A free energy difference of ~3 kcal/mol between charged and neutral hydrogen bonds has been found for binding to tyrosyl-tRNA synthetase.[97] A complete evaluation of the active site hydrogen bonds in citrate synthase with the AM1/CHARMM potential would require more extensive free energy calculations to be performed. However, interaction energies alone can provide insight for this system, shielded as it is from bulk solvent.

In CS, the acetyl-CoA enolate is stabilized at the active site by hydrogen bonds from His-274 and a conserved water molecule (Wat-585) as a consequence of these prexisting bonds becoming charged in the intermediate complex. As a proton is removed from acetyl-CoA, negative charge on the carbonyl oxygen increases, greatly strengthening the hydrogen bonds with His-274 and Wat-585. Although the energies of these bonds are smaller than some purely electrostatic interactions with neighboring charged groups in the enzyme, they offer the advantage of high specificity. In the terminology of Albery and Knowles,[98] the hydrogen bonds differentially stabilize the intermediate relative to the substrate. As these bonds bind the acetyl-CoA substrate, the entropic cost for their formation to stabilize the enolate or TS is lower than in solution, and this lowering of $\Delta G^{\ddagger}_{int}$ may also be significant in the rate acceleration, as noted above and proposed for preoriented dipoles in the active sites of other enzymes.[3] The hydrogen bond with His-274 also serves to bind and position acetyl-CoA, and rotation about the bond may guide the nucleophilic attack of the enolate upon oxaloacetate.[19]

Asp-375 Influences the Effective Stabilization of the Intermediate by His-274

The Marcus formalism analysis of the citrate synthase reaction mentioned above indicates that the nucleophilic intermediate is stabilized relative to the substrate ($\Delta G°$ is reduced) by 11.5–15 kcal/mol. It is therefore pertinent to ask whether the hydrogen bond donated by His-274 (Nδ1) to the carbonyl/enolate oxygen of acetyl-CoA can supply this amount of stabilization. Considerations of the hydrogen bond strengths in models of this interaction, including only methylimidazole (MeIm) and a thioester in its neutral (keto) or negatively charged (enolate) form, would seem to indicate that it can. The hydrogen bond between MeIm (representing His-274) and the enolate is very much stronger than the bond with the keto form of the thioester (representing acetyl-CoA). AM1 gives a hydrogen bond energy for the enolate/MeIm complex of 15.9 kcal/mol, 12.3 kcal/mol stronger than that in the neutral complex. AM1, and to a lesser extent PM3, do however underestimate the energies of strong hydrogen bonds somewhat.[65,99] PM3 gives a hydrogen bond energy for the enolate/MeIm complex of 19.2 kcal/mol, which is similar to the MP2/6-31+G(*d*)//RHF/6-31+G(*d*) interaction energy of 21.7 kcal/mol[62] (corrected for basis-set superposition error and scaled RHF/6-31+G(*d*) zero-point energies). PM3 gives a low hydrogen bond energy for the neutral complex of 2.3 kcal/mol, and therefore calculates the hydrogen bond to be 16.9 kcal/mol stronger in the enolate complex than in the keto complex.

These values apparently indicate that the greater strength of the enolate-His-274 hydrogen bond than the equivalent bond between acetyl-CoA and His-274 in the substrate complex could supply stabilization to the intermediate of the magnitude required. However, it is not always straightforward to define hydrogen bond energies because of the complications of secondary interactions. In this case, the most useful definition of the effective stabilization due to the hydrogen bond is its effect on the keto-enolate reaction, that is, the effect of the presence or absence of the hydrogen bond donor on the energy difference between the keto and enolate forms of acetyl-CoA *in the reacting system*. This effective stabilization cannot be gauged from measurements of the interaction energy of the two hydrogen-bonded components alone, but must include the other reacting species. The proximity of Asp-375, and its involvement in the reaction, significantly reduce the effective stabilization provided by the hydrogen bond with His-274. The roots of this effect can be thought of as favorable interactions with the carboxylate of Asp-375, particularly with (neutral) His-274, in the substrate complex, which are lost when Asp-375 is neutral (having abstracted a proton from acetyl-CoA) in the enolate

complex. Asp-375 should be included in any model to calculate the effective stabilization.

In complexes representing the proton abstraction from acetyl-CoA which included acetate/acetic acid to model Asp-375, the hydrogen bond was calculated to stabilize the enolate by only 5.3 kcal/mol (AM1) relative to the reactants. PM3 calculations on the reaction show the same effect: the hydrogen bond stabilizes the enolate by 7.4 kcal/mol. These values may be underestimated, but the agreement of the PM3 and ab initio hydrogen bond energies in the smaller complex mentioned above suggests that this is reasonable. It must be remembered that these energies are based on gas-phase interaction energies, and a full assessment of the catalytic value of the His-274 hydrogen bond would require its effect on the free energy along the whole reaction path to be evaluated. Many other groups in the enzyme also influence the relative stabilities of the different forms of the reacting system. Nonetheless, these results provide a strong indication that the effective stabilization of the enolate intermediate by the hydrogen bond from His-274 in CS, although highly significant, is much lower than the energy of the hydrogen bond in the gas phase.

Condensation With Oxaloacetate and Progress of the Reaction

The selection of His-274 as a neutral imidazole, instead of the much more acidic positively charged imidazolium or another type of amino acid residue is central to the CS mechanism, and bears further comment. The function of neutral histidine as the electrophilic or general acid catalyst in TIM (His-95) has been discussed in terms of avoiding inhibitory interactions with the carboxylate general base, matching the pK_a of the intermediate to maximize the rate (although calculations now indicate that the pK_as are not equal[22]), or avoiding the formation of an overstable neutral enediol intermediate, which would slow the overall reaction.[21,83] In CS, model calculations indicate that if His-274 were positively charged, no stable complex with the enolate could be formed. Instead, loss of a proton from His-274 would produce the enol form of acetyl-CoA. A reason for the importance of the enolate character of the intermediate is suggested by calculations on models of the condensation reaction with oxaloacetate.[64] Polarization at the active site will reduce the barriers to nucleophilic attack, but it is clear that the barrier for the addition of the enolate is very much lower than that for the enol, which is so high that it is not consistent with the observed rate, and would slow the overall reaction. Similarly, MNDO calculations on fatty acid biosynthesis[100] showed that the enolate, but not the enol, form of a thioester is capable of nucleophilic attack. As expected, the enolate is a much better nucleophile than the enol. For the formation of citrate to proceed rapidly, it is important that the nucleophilic intermediate resembles the enolate of acetyl-CoA. Hydrogen bonds with neutral His-274 and Wat-585 stabilize the charged intermediate, to preserve and harness its nucleophilic power.

CONCLUSIONS

Study of acetyl-CoA enolization in citrate synthase with the AM1/CHARMM QM/MM method has provided new insight into the enzyme mechanism, and the results are consistent with experimental and theoretical findings for CS and related enzymes. They support the assignment of Asp-375 act as the catalytic general base, but indicate that His-274 is neutral at the active site, and does not act as an acid. Rather, a hydrogen bond from this residue to the carbonyl oxygen of acetyl-CoA lowers the energy of the otherwise unstable charged enolate. The effective stabilization provided by this hydrogen bond (by 5.3 kcal/mol relative to the reactants, AM1; 7.4 kcal/mol, PM3) is not as large as has been suggested for strong hydrogen bonds in proteins (up to 20 kcal/mol). Interactions with Asp-375 at the active site are the cause of the effective stabilization at the active site being lower than the calculated increase in hydrogen bond energy in an isolated complex. The conditions for formation of a low-barrier hydrogen bond do not appear to be satisfied, as the basicities of the enolate and His-274 are calculated to be quite different. An ordered, conserved water molecule (Wat-585) plays a significant part in stabilizing the enolate (by 5.4 kcal/mol relative to the substrate, AM1/CHARMM), also through a hydrogen bond. Calculated residue contributions indicate that many groups in the enzyme contribute to the overall reaction energetics.

Together, the hydrogen bonds from Wat-585 and His-274 should provide specific stabilization of the enolate of approximately the magnitude estimated as necessary for CS from Marcus formalism analysis. These hydrogen bonds are formed in the substrate complex, and may therefore also assist in lowering the activation free energy relative to the solution reaction because of the resulting smaller solvation reorganization barrier.[3,25] The results indicate that differential stabilization[98] of the enolate relative to the substrate (i.e., reduction of ΔG°) is central to the catalytic strategy of CS, allowing it to achieve rapid deprotonation of its weakly acidic substrate. This strategy of enolate stabilization appears to be important for the next stage of the reaction, nucleophilic attack on the carbonyl carbon of oxaloacetate. The enolate of acetyl-CoA is a good nucleophile, and should react rapidly, whereas were the enol to be formed as an intermediate, it would react very much more slowly, and slow the rate of citrate formation.

This study has further demonstrated the useful contribution simulation approaches can make to the study of enzyme catalysis. As with any investigation employing an approximate potential, it is important

to consider limitations of the method, and this requires careful testing and application. Comparisons with more sophisticated calculations and experimental data are vital in this respect. Free energy relationships, such as Marcus formalism analyses of activation energies and other approaches,[101,102] can then be an informative link between experiment and theory in studies of this kind. When informed by and tested against experiment, simulations can aid in the interpretation and extension of results, and so add an extra dimension to studies of enzymic reactions.

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