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Ab initio QM/MM modelling of acetyl-CoA deprotonation in the enzyme citrate synthase

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This paper is dedicated to Prof. Graham Richards.

Abstract

The first step of the reaction catalysed by the enzyme citrate synthase is studied here with high level combined quantum mechanical/molecular mechanical (QM/MM) methods (up to the MP2/6-31+G(*d*)//6-31G(*d*)/CHARMM level). In the first step of the reaction, acetyl-CoA is deprotonated by Asp375, producing an intermediate, which is the nucleophile for attack on the second substrate, oxaloacetate, prior to hydrolysis of the thioester bond of acetyl-CoA and release of the products. A central question has been whether the nucleophilic intermediate is the enolate of acetyl-CoA, the enol, or an 'enolic' intermediate stabilized by a 'low-barrier' hydrogen bond with His274 at the active site. The imidazole sidechain of His274 is neutral, and donates a hydrogen bond to the carbonyl oxygen of acetyl-CoA in substrate complexes. We have investigated the identity of the nucleophilic intermediate by QM/MM calculations on the substrate (keto), enolate, enol and enolic forms of acetyl-CoA at the active site of citrate synthase. The transition states for proton abstraction from acetyl-CoA by Asp375, and for transfer of the hydrogen bonded proton between His274 and acetyl-CoA have been modelled approximately. The effects of electron correlation are included by MP2/6-31G(*d*) and MP2/6-31+G(*d*) calculations on active site geometries produced by QM/MM energy minimization. The results do not support the hypothesis that a low-barrier hydrogen bond is involved in catalysis in citrate synthase, in agreement with earlier calculations. The acetyl-CoA enolate is identified as the only intermediate consistent with the experimental barrier for condensation, stabilized by conventional hydrogen bonds from His274 and a water molecule.

Keywords: QM/MM; Citrate synthase; Enzyme catalysis; Low barrier hydrogen bond; Proton transfer; Enolate intermediate

1. Introduction

Achieving a deeper understanding of enzyme catalytic processes is a problem of great practical and fundamental significance. Computer simulations can make an important contribution by providing a description of enzyme mechanisms at the molecular level [1–4]. Calculations can be used to study unstable species, to evaluate possible alternative mechanisms, and to calculate energetic contributions to catalysis. These are central considerations in an enzyme-catalysed reaction, which are difficult to address by experiment alone. A first step in the modelling process is the investigation of possible reaction intermediates and transition states (TSs), to identify basic features of the potential energy surface governing the reaction. One approach is to perform 'supermolecule' calculations on clusters of small molecular fragments representing functional groups of the enzyme and substrate [5–7] by standard quantum chemical techniques. The structures of TSs and stable complexes can then be optimized. By necessity, usually only a small portion of the enzyme can be treated (although new approaches allow quantum mechanical calculations on whole proteins [8–13]). The surrounding protein and solvent is likely to have a significant effect on the reaction and ideally should be represented. Furthermore, from a practical point of view, it is often difficult to perform geometry optimizations in such supermolecule calculations, because it can be difficult to apply constraints which realistically mimic the covalent and non-bonded interactions at the active site but at the same time are

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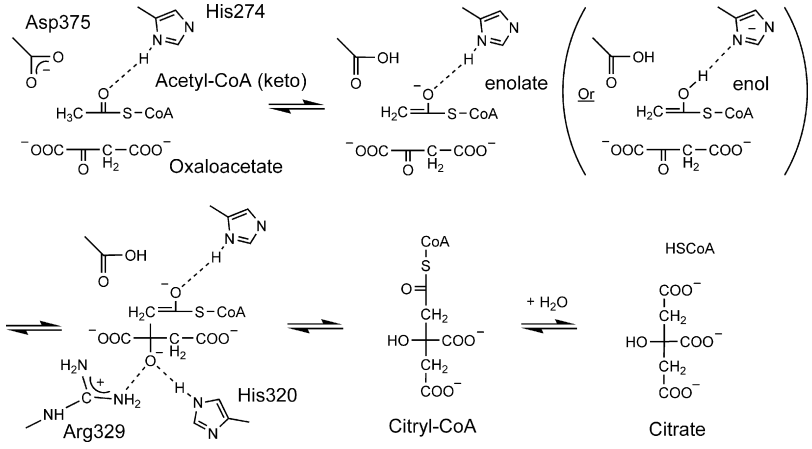
flexible enough to allow optimization. Optimization without constraints can lead to the fragments drifting in ways which would be impossible in the confines of the active site, producing complexes which are not relevant to the reaction in the enzyme. One way to overcome these problems is to use combined quantum mechanical/molecular mechanical (QM/MM) methods, a now widely used approach. The essence of the QM/MM approach is that a small region is treated quantum mechanically, and is coupled to a simpler molecular mechanics description of the remainder of the system [14]. QM/MM calculations have given useful insight into a large number of enzyme reactions (e.g. [3,15]). Before around the year 2000, most applications to enzyme reactions were at the semiempirical molecular orbital (MO) level of QM treatment, due to the demands of higher level calculations. Semiempirical methods offer the advantage of being highly computationally efficient, which allows molecular dynamics simulations to be performed, and the free energy profile to be calculated [16,17]. They are, however, also subject to errors in some cases, making it important to test the reliability of the semiempirical methods for a given application. Ab initio or DFT QM/MM calculations on reactions in enzymes have become increasingly popular in recent years, resulting in often successful reproduction of experimental results [15,18] and insightful information on enzymatic reaction mechanisms [19]. Recently, it has become possible to apply even high-level local correlation methods in QM/MM calculations on enzyme reactions, though such computations remain highly demanding [18].

We have carried out ab initio QM/MM calculations on the reaction catalysed by citrate synthase to test conclusions drawn from earlier studies which used a semiempirical QM treatment [20], and to study the performance of the ab initio QM/MM method. Citrate synthase catalyses the formation of citrate from acetyl-coenzyme A (acetyl-CoA) and oxaloacetate in the citric acid cycle [21,22]. The reaction proceeds via deprotonation of acetyl-CoA to form a nucleophilic intermediate [6,23,24], which subsequently attacks the carbonyl carbon of oxaloacetate, which is polarized at the active site [25,26]. Hydrolysis of the thioester bond then allows release of the products. Crystallographic and mutagenesis experiments have identified Asp375 as the catalytic base [27,28], with His274 also observed to interact with acetyl-CoA [29] and play a catalytic role [30] (the numbering used is that for pig citrate synthase). This mechanism appears to be conserved across all (*S*-)citrate synthases [31,32]. It has been uncertain whether the nucleophilic intermediate is the enolate or enol of acetyl-CoA (Scheme 1) [33], or alternatively an 'enolic' form, sharing a proton with His274 in a 'low-barrier' hydrogen bond [34,35]. This is an important question, relating directly to the mechanisms used by this and other enzymes to stabilize reaction intermediates and attain rapid reaction rates [20]. It has been proposed that the enolate is too unstable to be consistent with the observed rate of reaction, unless it is significantly stabilized by the enzyme [36]. However, the lack of an effective general acid at the active site means that the enol form of acetyl-CoA appears to be similarly unstable. Citrate synthase is not dependent on metal ions for catalysis [21,22], and so the stabilization of any intermediate must be due to interactions with the protein itself. One explanation put forward is that the intermediate in citrate synthase (and unstable intermediates in many other enzymes) is stabilized by a low-barrier hydrogen bond [34,35,37], but this proposal has been highly controversial [38–42].

In the present work, we focus on the energies of the substrate and postulated intermediate forms of acetyl-CoA bound at the active site of citrate synthase. The calculations build on earlier semiempirical QM/MM studies [20], and on QM calculations on proton transfer in gas-phase models [43]. The results provide no support for the proposal that a low-barrier hydrogen bond stabilizes the intermediate in citrate synthase. Normal hydrogen bonds from His274 and an active site water molecule stabilize the charged enolate significantly relative to bound substrate. The enolate form of acetyl-CoA is calculated to be clearly more stable than the enol or enolic forms, and is the probable nucleophilic intermediate.

2. Methods

The starting points for the calculations were the structures of the keto (substrate), enolate and enol forms of acetyl-CoA



Scheme 1. Reaction mechanism of citrate synthase, indicating the substrate (keto) and possible intermediate (enolate and enol) forms of acetyl-CoA.

bound to chicken citrate synthase produced by semiempirical QM/MM calculations [20]. The simulation system was generally as described for the earlier calculations [20], with some differences as noted below. Briefly, it consisted of a 17 Å radius sphere, containing 1717 atoms, around the active site taken from a high-resolution crystal structure of chicken citrate synthase complexed with acetyl-CoA and the inhibitor *R*-malate [29]. Atoms between 14 Å and 16 Å from the active site were harmonically restrained to their positions in the crystal structure, and those further than 16 Å away were kept fixed. The structures of the substrate complex (the keto form of the acetyl-CoA thioester), and the enolate and enol (produced by reaction pathway calculations) were energy minimized (500 steps steepest descents followed by 1000 steps Powell conjugate gradients), with the reacting groups treated quantum mechanically at the AM1 level. 'United atom' parameters were used for the MM atoms [44], with atomic charges for the substrates as given previously [20]. No modifications were made to the MM van der Waals parameters for the QM atoms.

The CHARMM program [45] was used for QM/MM calculations. Testing and implementation of the QM/MM method (at the semiempirical level) has been detailed previously [14]. CHARMM has also been interfaced with the quantum chemical package GAMESS-US [46] to allow QM/MM calculations with an ab initio MO treatment for the QM region [47]. In this QM/MM approach, the atomic charges of the MM atoms are included in the QM Hamiltonian to calculate energies and forces, and the two regions also influence each other through MM bonded and non-bonded terms. Where the boundary between the QM regions separates covalently bonded atoms, a 'link' (hydrogen) atom is used to fulfill the bonding requirements of the QM system. In the present case, the sidechains of Asp375 and His274, the thioester tail of acetyl-CoA, and a water molecule were treated quantum mechanically. This is somewhat different to the earlier semiempirical calculations: a slightly smaller part of acetyl-CoA was included here in the QM region (CH_3COSCH_2–R, equivalent to methylthioacetate (R is replaced by a hydrogen atom in the calculations)). The present partitioning allows direct comparison to standard ab initio calculations on complexes, which used the same molecules to represent the enzyme reaction [43]. In addition, it was found to be preferable to expand the QM region to include a water molecule, which was identified by earlier analysis as influencing the reaction by hydrogen bonding to the carbonyl oxygen of acetyl-CoA [20]. This water is designated Wat585 (from the numbering used in the Brookhaven Protein Databank file 4CSC.PDB [29]), and is part of a cluster of three ordered water molecules observed crystallographically at the active site in citrate synthase ternary complexes. It was found that while the overall reaction energetics were similar with Wat585 treated QM or MM, the hydrogen bond from this water to the acetyl-CoA oxygen was shorter in all the complexes by approximately 0.3 Å when it was treated MM. This interaction is potentially significant, and so the more accurate QM treatment of Wat585 was preferred, giving a total of 33 QM atoms. (The other two water molecules from the cluster do not interact directly with the reacting species and were therefore not included in the QM region.) The details of the QM/MM partitioning are also slightly different in the present study. The 'link' atoms are treated in the same way as all the other QM atoms in terms of their interactions [47]. All the QM atoms interact with all the MM atoms, except the MM groups bonded to QM atoms. As previously, no non-bonded cut-off was used. A preliminary account of related calculations has been published previously [48]. Here, we extend the level of QM treatment and analyse the structures and energetics in detail.

Initially, energy minimization was at the RHF/3-21G(*d*) QM/MM level, and then extended to the RHF/6-31G(*d*) level. The prior optimization carried out at the AM1/CHARMM level was important to sufficiently minimize the MM region, because the computational demands of the ab initio QM/MM method limited minimizations to the order of 100 steps. This is sufficient for the QM system to attain a good geometry (as shown by comparison to fully optimized complexes [43]) and for the relaxation of the MM region to accommodate the relatively small electronic and structural changes at the active site on going to the higher level. Fifty steps of steepest descent (SD) minimization were performed for each of the keto, enolate and enol forms at the 3-21G(*d*) level, followed by 100 steps with the Powell conjugate gradient method [45]. For the enol, Powell minimization resulted in proton transfer, and so the structure after SD minimization only was used. Twenty-five steps of SD followed by 85 steps of Powell conjugate gradients were then carried out for each structure at the 6-31G(*d*) QM/MM level. Throughout, all atoms were free to move, except at the boundaries of the simulation sphere where restraints were applied as described above. For simplicity, we refer throughout to the complexes along the reaction pathway by the form of acetyl-CoA involved, namely the keto (substrate), enolate or enol.

The TS structures for proton transfer were modelled approximately by restraining the proton to be equidistant between the exchanging heavy atoms during minimization, with a harmonic force constant of 1000 kcal/mol $Å^2$. The two transfers considered are the abstraction of an acetyl methyl proton from acetyl-CoA by Asp375 (keto to enolate reaction, Scheme 1) and protonation of the enolate oxygen by His274 (enolate to enol reaction). The advantage of this approximate approach is that only first derivatives of the energy are required. The similar energies of the keto and enolate complexes in vacuo, and comparison with fully characterized TS structures [43] show that it is a reasonable approximation in the case of the keto to enolate reaction. It is less good for modelling the TS for the conversion of the acetyl-CoA enolate to the enol, as the enol is very much higher in energy. The structure with the proton equidistant between the hydrogen bonding N and O atoms (of His274 and the acetyl-CoA enolate, respectively) is of interest as representing the postulated enolic intermediate [35,37] in which the proton is shared between them. The sensitivity of the results to the choice of starting structure was tested by modelling each TS starting from the fully minimized reactant and product complexes for the relevant proton transfer, resulting in two TS models for each step. These are referred

to as TS1a and TS1b for the keto–enolate reaction (produced starting from the keto and enolate complexes, respectively), and TS2a and TS2b as models of the enolic intermediate (starting from the enolate and enol complexes, respectively). The resulting structures and energies were similar. Each model was minimized (50 steps SD followed by 60 steps Powell conjugate gradients) at the 6-31G(*d*) QM/MM level. Minimization of the keto, enolate and enol forms and of the 4 TS models were sufficiently converged; RMS gradients were about 0.1 kcal mol^{-1} $Å^{-1}$ for all seven structures (see supporting information).

MP2/6-31G(*d*) and MP2/6-31+G(*d*) calculations were performed on the QM/MM energy minimized geometries to assess the effects of electron correlation on the QM system. Gaussian-94 [49] was used for these calculations, which included only the QM atoms in the geometries resulting from QM/MM optimization. The net charge of the QM system was −1. In treating anionic systems it is generally preferable to include diffuse functions [46,49]. However, it was found that SCF convergence problems occurred when diffuse functions were used in QM/MM minimizations and single point energy calculations. This is due to interactions with MM groups, as RHF/6-31+G(*d*) and MP2/6-31+G(*d*) calculations treating the QM system only converged normally. Further analysis will be required to establish the causes of this behaviour. Comparison of representative complexes showed geometries optimized with and without diffuse functions are very similar [43], so that in this case a good description of the geometry of the reacting system can be achieved without the use of diffuse functions. The energetic effects on the QM system of extending the basis set for the keto, enolate and enol complexes were studied by MP2/6-31+G(*d*) and RHF/6-31+G(*d*) single point calculations.

3. Results

Fig. 1 shows the energy profile for abstraction of a proton from acetyl-CoA by Asp375 in citrate synthase (described here as the keto to enolate reaction), and for proton transfer from His274 to the enolate oxygen to form the enol, at the 6-31G(*d*) QM/MM level. The energy of the QM atoms alone at the same level is also shown. The enolate form of acetyl-CoA is calculated to be clearly more stable than the enol at the active site of citrate synthase. It is markedly unfavorable for His274 to donate a proton to acetyl-CoA. The structures representing the enolic intermediate (with the hydrogen bonded proton equidistant between ND1 of His274 and the enolate oxygen) are also of significantly higher energy than the enolate at all theoretical levels studied here.

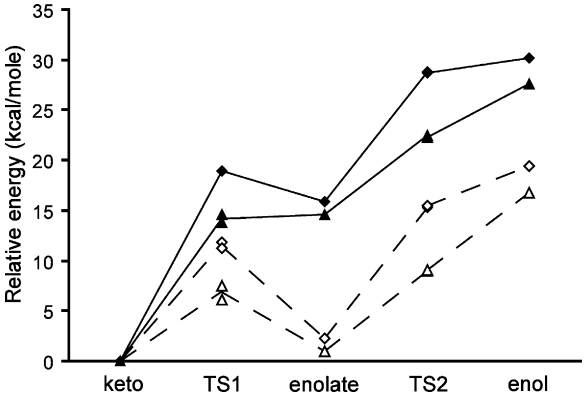


Fig. 1. Energy profile for acetyl-CoA enolization in citrate synthase showing the QM/MM energy for the reaction in the protein (solid line), and that of the QM system alone (dashed line), at the RHF/6-31G(*d*) (diamonds) and MP2/6-31+G(*d*) levels (triangles). Energies are shown relative to that of the keto (substrate) form of acetyl-CoA in each case.

The structures produced by ab initio QM/MM minimization are generally very similar to those from the earlier semi-empirical calculations [20]. No significant changes are apparent in the MM region, although in the vicinity of the active site, many atoms show small shifts in position in adjusting to electronic and structural changes at the active site. The basic features of the QM system also remain unchanged, but notable changes in its internal geometry (e.g. in hydrogen bond distances between QM atoms) are found. The structures of the keto and enolate forms of acetyl-CoA bound at the active site of citrate synthase are shown in Fig. 2, and some important interatomic distances are given in Table 1. In the substrate (keto) complex, His274 and Wat585 donate hydrogen bonds to the acetyl carbonyl oxygen of acetyl-CoA. These hydrogen bonds are shorter in the enolate complex, indicating that they become stronger upon deprotonation of the substrate. Conversion of the enolate to the enol involves transfer of the hydrogen bonding proton from ND1 of His274 to the oxygen of the substrate. In the enol complex, and models of the enolic intermediate, the His274(ND1)-(O)acetyl-CoA distance is shorter still (see Fig. 2).

One QM/MM interaction of note is a hydrogen bond donated by the sidechain of Ser244 to NE1 of His274, which shortens slightly on going from the keto (O···N = 2.81 Å, OH···N = 1.86 Å) to the enolate (O···N = 2.81 Å, OH···N = 1.85 Å) to the enolic and enol forms (O···N = 2.79 Å, OH···N = 1.83 Å), as negative charge increases on the sidechain of His274. The variation in this interaction shows that electronic changes at the active site coincide with changes in the conformation of the surrounding protein, demonstrating the importance of allowing the MM region in the vicinity to move freely during energy minimization. The mainchain NH group of Ser244 is also positioned close to NE1 of His274, but in a poorer geometry for hydrogen bonding (N···N = approximately 2.9 Å in all three complexes). Ser244 was identified through analysis of QM/MM interaction energies as possibly important for stabilizing the enol complex, and has been found to be conserved in citrate synthase from a wide variety of species [20]. The grouping of the acetyl-CoA carbonyl, His274 and Ser244 has some similarity to the (Asp-His-Ser) catalytic triad of the serine proteases [20]. The hydrogen bond between the sidechains of His274 and Ser244 stabilizes the enol and enolic forms of acetyl-CoA to some extent, as in these forms partial or complete proton transfer from ND1 of His274 to acetyl-CoA results in negative charge accumulating on the imidazole (imidazolate) ring. However, this stabilization is calculated to be insufficient to make the enol or enolic forms of comparable energy to the enolate. Although a complete description would

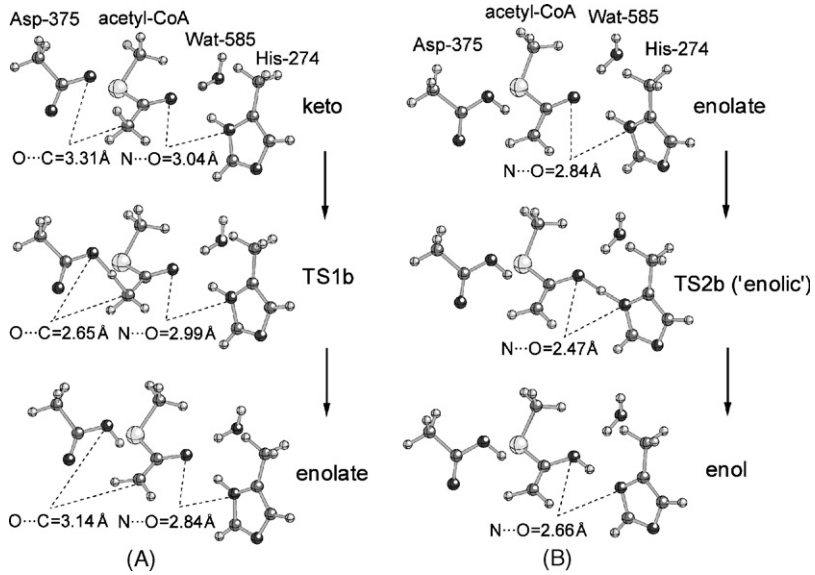


Fig. 2. (A) Structures of the keto (substrate), approximate TS and enolate forms of acetyl-CoA and (B) structures of the enolate, 'enolic' and enol forms of acetyl-CoA bound at the active site of citrate synthase after RHF/6-31G(*d*) QM/MM energy minimization.

require Ser244 to be treated quantum mechanically, the best indications are that taking the effects of this interaction into account the conventionally hydrogen bonded enolate of acetyl-CoA is the likely intermediate. The conservation of Ser244 shows that it plays an important role, which may be to position His274 correctly for substrate binding, and to ensure the neutrality of the histidine sidechain. Cooperative interactions [50] between Ser244 and His274 may also strengthen the hydrogen bond with the enolate intermediate.

Ab initio calculations on proton transfer between methylthioacetate and acetate, and between the enolate of methylthioacetate and methylimidazole in isolation have been carried out previously, with TS and stable geometries characterized by normal mode analysis [43]. Comparison with these fully optimized structures shows that the structures produced by QM/MM minimization are reasonable. The inter- and intra-molecular geometrical variables are similar. For example, the heavy atom separation at the TS model for abstraction of a proton by Asp375 (2.65 Å (TS1a, TS1b)) is the same as that in the RHF/6-31G(*d*) TS for this process. Similarly, the QM/MM model TSs for proton exchange along the His274-acetyl-CoA hydrogen bond have N···O distances of 2.47 Å and 2.48 Å, compared to 2.48 Å for the bimolecular reaction. It should be remembered that the TS models in this study were optimized subject only to the restraint that the heavy atom–proton distances should be equal. They are only approximate models, but it seems likely that the separation of the exchanging heavy atoms in each case is close to that in the exact TS. For example, stabilization of the enolate makes the O–H and C–H distances more similar (in line with Hammond's postulate), but leaves the heavy atom separation virtually unchanged [43]. The TS models obtained using different starting structures (i.e. TS1a/TS1b and TS2a/TS2b) show minor structural differences. The calculated QM/MM energies in each case are similar, however, indicating that the results are not very sensitive to these differences.

Table 1
Some important interatomic distances in the citrate synthase active site from RHF/6-31G(*d*) QM/MM minimized structures

Description	Keto	TS1a	TS1b	Enolate	TS2a	TS2b	Enol
Asp375-AcCoA[a]							
OD1···C	3.31	2.65	2.65	3.14	3.30	3.37	3.45
OD1···H	2.24	1.35	1.34	0.97	0.96	0.96	0.96
H···C	1.09	1.32	1.32	2.23	2.41	2.52	2.65
His274-AcCoA[b]							
ND1···O	3.04	3.04	2.99	2.84	2.48	2.47	2.66
HD1···O	2.05	2.05	2.00	1.83	1.25	1.24	1.00
ND1···HD1	1.00	1.01	1.00	1.01	1.23	1.23	1.67
Wat585-AcCoA[c]							
O···O	2.95	2.96	2.95	2.81	2.84	2.85	2.91
(HO)H···O	2.01	2.02	2.01	1.85	1.89	1.90	1.96
(HO)–H	0.95	0.95	0.95	0.96	0.95	0.96	0.95

[a] Atoms involved in transfer of proton (H) from acetyl-CoA acetyl methyl carbon (C) to OD1 of Asp375.
[b] Hydrogen bond between ND1 of His274 and acetyl oxygen (O) of acetyl-CoA.
[c] Hydrogen bond donated by Wat585 to acetyl oxygen of acetyl-CoA.

One of the most important features at the active site is the hydrogen bond between ND1 of His274 and the acetyl oxygen of acetyl-CoA, which is central to stabilizing the intermediate resulting from deprotonation of the substrate. In the enolate complex, this hydrogen bond is noticeably shorter than in the substrate complex, with an N···O distance of 2.84 Å, similar to that in the isolated enolate-methylimidazole complex (2.77 Å, 6-31G(*d*) [43]). The slightly longer bond in the enzyme compared to the isolated bimolecular complex is probably due to cooperative interactions with Wat585, which are known to be important in hydrogen bonded systems [50]. In the enol complex, in which the proton has been transferred from His274 to the oxygen of acetyl-CoA, the hydrogen bond is shorter still (N···O = 2.66 Å), and marginally shorter than in the equivalent bimolecular complex (N···O = 2.69 Å, 6-31G(*d*) [43]). The TS models for proton transfer along the hydrogen bond are clearly not the true TS, as they are lower in energy than the products. However, the heavy atom separation is very close to that of the exact TS as noted above. These are good models of the potential 'enolic' intermediate, in which the proton is shared between the heavy atoms in a low-barrier hydrogen bond, proposed for this enzyme [35,37]. The heavy atom separation in the enolic models (ND1···O = 2.48 Å (TS2a), 2.47 Å (TS2b)) is similar to that found in the shortest, proton sharing N···O hydrogen bonds (N···O = 2.5 Å approximately) [51].

Having established that the geometries produced by QM/MM minimization are well optimized, the energies of the system can be examined in more detail. Analysis of the total energy change for the two reaction steps (Table 2) shows that the largest contribution to the overall energy barrier and energy changes for reaction is due to the energy of the QM system, as expected (the QM/MM energy listed is the energy of the QM system including the electrostatic effects of the MM atoms). Smaller changes in the energy of the MM system do also affect the total energy profile however. Removal of a proton from acetyl-CoA to form the enolate is accompanied by a small increase in the van der Waals energy (which includes interactions of QM atoms with MM atoms), together with a larger decrease in the electrostatic energy of the MM system. The net effect is to stabilize the enolate relative to the substrate (keto) complex; the same effect also stabilizes the enol and enolic forms. A small (∼1 kcal/mol) change in internal energy (from MM bonded terms) also favors the enolate, enolic and enol complexes over the substrate complex. These contributions mean that the increases in total energy on going from the keto to the enolate or enol forms are smaller than the increases in the QM energy. In separate 6-31G(*d*) QM/MM minimizations treating Wat585 MM, this effect was not apparent, and the relative total energies along the reaction pathway were similar in both cases. With Wat585 treated MM, the enolate was 15.7 kcal/mol higher in total energy (15.8 kcal/mol, QM/MM), and the enol was 30.3 kcal/mol higher (30.5 kcal/mol, QM/MM) than the keto form.

It is worth noting that the structures produced by optimization with and without diffuse functions (RHF/6-31G(*d*), RHF/6-31+G(*d*)) are very similar [43], showing that diffuse functions are not required to give good geometries for this system. This means that single point calculations at the higher level (Table 3) can give useful results. Addition of diffuse functions, which will improve the description of these systems, increases the energy of all forms relative to the keto complex (by approximately 3–4 kcal/mol). Single point calculations on the optimized QM geometries were also used to examine the effects of electron correlation, as it was not practical to carry out correlated QM/MM calculations. Comparing the RHF energies with those calculated at the MP2 level, it is clear that electron correlation significantly affects the reaction energetics (see also Fig. 1). At the MP2/6-31G(*d*) level, the enolate form is more stable even than the substrate (3.2 kcal/mol below the keto form) when the QM atoms alone are considered. The keto and enolate forms are also of comparable energy at the MP2/6-31+G(*d*) level (enolate 1.0 kcal/mol above the keto form). As expected, the effects of electron correlation are largest for the TS models. The approximate barriers to the keto–enolate reaction (for the

Table 2
QM/MM energies for acetyl-CoA enolization in citrate synthase relative to bound substrate (keto form of acetyl-CoA), indicating contributions to the total calculated energy

	Keto	TS1a	TS1b	Enolate	TS2a	TS2b	Enol
Total energy	0.0	18.9	18.9	15.9	28.8	28.7	30.2
QM/MM 6-31G(*d*)[a]	0.0	22.0	22.0	22.0	35.2	35.2	36.3
VDW[b]	0.0	0.2	1.7	2.3	2.0	2.2	2.2
ELEC[c]	0.0	−3.2	−4.6	−7.4	−7.5	−8.2	−7.7
Internal (MM)[d]	0.0	−0.5	−0.6	−1.1	−1.2	−0.9	−0.7
ΔMP2/6-31G(*d*)[e]	0.0	−7.9	−8.8	−5.5	−10.9	−10.8	−6.7
Total + MP2[f]	0.0	11.0	10.1	10.4	17.9	17.9	23.5
ΔMP2/6-31G+(*d*)[e]	0.0	−4.3	−5.1	−1.3	−6.3	−6.4	−2.6
Total + MP2+[f]	0.0	14.6	13.8	14.6	22.5	22.3	27.6

[a] QM/MM electronic energy (energy of QM system including effects of MM partial charges).
[b] MM van der Waals energy, including QM/MM interactions.
[c] MM electrostatic energy.
[d] MM internal energy.
[e] MP2/6-31G(*d*) or MP2/6-31G+(*d*) correction for QM energy only, relative to that for keto form.
[f] Total energy plus MP2/6-31G(*d*) or MP2/6-31G+(*d*) correction.

Table 3
Ab initio and semiempirical energies for QM atoms only, relative to the keto form of acetyl-CoA

	Keto	TS1a	TS1b	Enolate	TS2a	TS2b	Enol
All QM atoms (including Wat585)							
RHF/6-31G(*d*)	0.0	11.8	11.2	2.3	15.3	15.5	19.4
MP2/6-31G(*d*)	0.0	3.9	2.4	−3.2	4.4	4.7	12.7
RHF/6-31+G(*d*)	0.0	14.7	14.3	5.9	19.4	19.5	22.7
MP2/6-31+G(*d*)	0.0	7.5	6.1	1.0	9.0	9.1	16.8
'Asp375, His274 and acetyl-CoA' only							
RHF/6-31G(*d*)	0.0	13.0	12.8	7.6	18.8	19.0	20.8
MP2/6-31G(*d*)	0.0	5.5	4.2	2.5	8.5	8.7	14.3
RHF/6-31+G(*d*)	0.0	15.7	15.7	10.8	22.7	22.8	24.2
MP2/6-31+G(*d*)	0.0	8.8	7.8	6.5	13.1	13.2	18.7
AM1[a]	0.0	11.7		2.4	15.4		12.5
AM1[b]	0.0	8.5		0.8	14.1		11.0

Ab initio energies calculated for 6-31G(*d*) QM/MM energy minimized geometries, with and without Wat585 included, with methylimidazole and acetate representing the sidechains of His274 and Asp375, respectively, and methylthioacetate representing acetyl-CoA.
[a] Semiempirical energies calculated for same QM model, with all complexes fully optimized as TSs or stable geometries [43].
[b] Semiempirical energies calculated for QM system from earlier AM1 QM/MM calculations in which ethylthioacetate represented acetyl-CoA [20].

QM system alone) are reduced to 2.4–3.9 kcal/mol, MP2/6-31G(*d*) and 6.1–7.5 kcal/mol MP2/6-31+G(*d*). The enolic structures TS2a and TS2b are also stabilized at the correlated level, but remain approximately 8 kcal/mol higher in energy than the enolate at the MP2 level with both basis sets. It is highly unfavorable to transfer the hydrogen bonded proton to form the enol. The MP2 energies are more sensitive to the small differences in geometry between TS models TS1a and TS1b, and between the enolic structures TS2a and TS2b, but the differences in energy are still small and do not affect these findings.

The effects of the surrounding protein, substrate and solvent groups (that together make up the MM region) on the energy profile can be seen by comparing the RHF/6-31G(*d*) results for the QM system in isolation (Table 3 and Fig. 1) with the QM/MM results (Table 2 and Fig. 1). Including the effects of the MM surroundings results in marked destabilization of the enolate (by 19.7 kcal/mol) and enol (by 16.9 kcal/mol) relative to the keto complex. The TS models TS1a and TS1b are also destabilized by the MM atoms relative to the substrate complex (by 10.2–10.8 kcal/mol, TS1a, TS1b), as are the enolic structures (by 19.8–19.9 kcal/mol, TS2a, TS2b). The 6-31G(*d*) energy difference between the enolate and enol is lowered somewhat (from 17.1 to 14.3 kcal/mol) through interactions with the MM system, but remains large. Detailed analysis of the QM/MM interactions [20,52–54] is required to identify individual MM groups that significantly affect the calculated energetics. Residue contributions calculated previously for citrate synthase showed that the second substrate, oxaloacetate, has the largest effect in destabilizing the enolate [20]. This can be understood as oxaloacetate bears a large negative charge and is bound close to acetyl-CoA. Deprotonation of acetyl-CoA by Asp375 causes negative charge to accumulate on the enolate, which is disfavored by the proximity of dianionic oxaloacetate. Oxaloacetate is known to be polarized when bound to citrate synthase [25,26], and it may be that atomic charges derived from a fit to the gas phase electrostatic potential for this molecule are not a good representation, and charges reflecting this polarization should be used instead. Optimization of van der Waals parameters to fit high level interaction energies and geometries could also be required. Alternatively, the intimate involvement of oxaloacetate in the reaction may mean that a simple MM point charge representation is inadequate for this group. It has been found that the barrier and reaction energy for proton transfer in water clusters is different when bound waters are represented by the SPC point charge model than when they are treated quantum mechanically in density functional calculations [55]. It is notable that the QM/MM energies of the TS models are very similar to the QM/MM energies of the products of each step. TS1a and TS1b have the same QM/MM energy as the enolate complex, despite the fact that the energies of the QM atoms alone are considerably higher for the TS models. Similarly, TS2a and TS2b have QM/MM energies close to that of the enol. This could perhaps be evidence of excessive QM/MM repulsion. Comparison with interaction energies calculated fully quantum mechanically, would be required to gauge the accuracy of the QM/MM interaction energies. Ideally oxaloacetate should be treated quantum mechanically, as it is involved in the next step of the reaction, but this was not computationally feasible at the time the calculations were carried out. Generally it is found, however, that interactions between QM and MM groups are calculated accurately by the method employed here [47]. It is clear that nearby groups destabilize the enolate and enol products of the proton transfers modelled here. Other groups in the protein were also found previously to have significant effects on the reaction, particularly charged groups at the active site [20]. Dielectric screening has not been included in the present study, and so electrostatic interactions, particularly with more distant groups, will be overestimated, but this does not qualitatively affect the energy profile for reaction. This, and other factors, such as the uncertain charge state of His238, (which binds oxaloacetate, and is treated here as positively charged), limit the accuracy of the calculations, but do not affect the conclusions qualitatively [20]. The fact that many groups around the active site influence

the reaction energetics stresses the need to include such groups in any full treatment of an enzyme reaction potential surface.

Adding the effects of electron correlation to the QM/MM energies is likely to give a more accurate picture of the overall reaction energetics, and the MP2 corrections relative to that for the keto complex are shown in Table 2 and Fig. 1. The approximation is made that the correlation correction is applied to the QM system only, rather than to the QM/MM energy. Including MP2/6-31G(*d*) corrections does not change the conclusion that the enolate form is significantly lower in energy than either the enolic or enol forms of acetyl-CoA at the active site of citrate synthase. The protein, which has been energy minimized to adopt a favorable conformation around each form, does not stabilize the enol significantly relative to the enolate, and the energy difference between these forms remains large when the effects of the protein and electron correlation are included (enol 12.1 kcal/mol higher in energy). This is an important point, relating to the proposal that equalization of the pK_a's of an active site acid with an unstable intermediate might be an important general mechanism of enzyme catalysis [35,37]. If this mechanism were important in citrate synthase, it would be expected that interactions with other groups would make His274 more acidic, and that the energies of the enolate and enol forms would be similar at the active site. The calculations do not support this proposal. The energy difference between the enolate and enol forms is large at all levels (as expected given the difference in acidity between the enol and imidazole), and it is not significantly reduced when the effects of the surroundings are considered. This indicates that the effective pK_a's of the intermediate and His274 are not equal at the active site, and so the conditions for formation of a low-barrier hydrogen bond are not met.

The models of the strongly hydrogen bonded complex (TS2a and TS2b), with a proton shared between His274 and the acetyl-CoA enolate, are also of considerably higher energy than the conventionally hydrogen bonded enolate even when the effects of the environment and/or MP2 correlation corrections are included. Both models of the proposed enolic intermediate lie 6.5 kcal/mol above the enolate (QM/MM+MP2/6-31G(*d*)), this energy difference reduced only marginally (by 1.1–1.4 kcal/mol) from that in the gas phase. Taken together, these results provide no support for the proposal that the hydrogen bond between His274 and acetyl-CoA is of the low-barrier type in the intermediate complex. Instead, the enolate of acetyl-CoA, stabilized by conventional hydrogen bonds from His274 and an active site water molecule, is identified as the likely nucleophilic intermediate.

It could be argued that the presence of Wat585 in the QM system disfavors the enol or strongly hydrogen bonded enolic forms by preferentially stabilizing the enolate, although it is recognized that low-barrier hydrogen bonds can exist in the presence of ordered water [56]. In all the minimized structures, the water molecule donates a hydrogen bond to the acetyl-CoA oxygen. Deleting the water molecule and recalculating the energies shows that the enolate is still definitely of lower energy than the enolic or enol forms (Table 3). The energies of the enolic models and that of the enol relative to the substrate increase slightly on deletion of the water molecule. Comparison of the energies along the reaction path with and without Wat585 present gives an approximate indication of its contribution to the overall energetics (Table 4 and Fig. 3). Similarly, the approximate contribution of His274 to stabilization of the enolate can be calculated by comparing the reaction profiles with this group present or not. These two hydrogen bonds, which are calculated to be of the conventional type with the protons localized on the donor heavy atom in each case, significantly stabilize the enolate relative to the keto form (by 17.1 kcal/mol, MP2/6-31+G(*d*)). His274 makes a larger

Table 4
Approximate contributions of the sidechain of His274 (represented by methylimidazole) and Wat585 to stabilizing the enolate of acetyl-CoA, relative to the keto (substrate) form, from single point calculations on 6-31G(*d*) QM/MM optimized geometries

Method	Stabilization relative to keto form (kcal/mol)
'His274' (methylimidazole)	
RHF/6-31G(*d*)	11.6–10.7
MP2/6-31G(*d*)	13.1–12.3
RHF/6-31+G(*d*)	10.4–9.6
MP2/6-31+G(*d*)	11.6–11.2
AM1[a]	5.3
Wat585	
RHF/6-31G(*d*)	5.3–6.2
MP2/6-31G(*d*)	5.7–6.5
RHF/6-31+G(*d*)	4.9–5.7
MP2/6-31+G(*d*)	5.5–5.9
AM1/TIP3P[b]	5.4
'His274 + Wat585'	
RHF/6-31G(*d*)	16.9
MP2/6-31G(*d*)	18.8
RHF/6-31+G(*d*)	15.3
MP2/6-31+G(*d*)	17.1

Contributions are calculated from the change in energy for the keto to enolate reaction resulting from deletion of the group in question. Results are given for the stabilization resulting from methylimidazole and Wat585 with and without the other present.
[a] AM1 QM/MM optimized geometry with same QM model [43].
[b] AM1 QM/MM optimized geometry with acetyl-CoA respresented by ethylthioacetate in QM model [20].

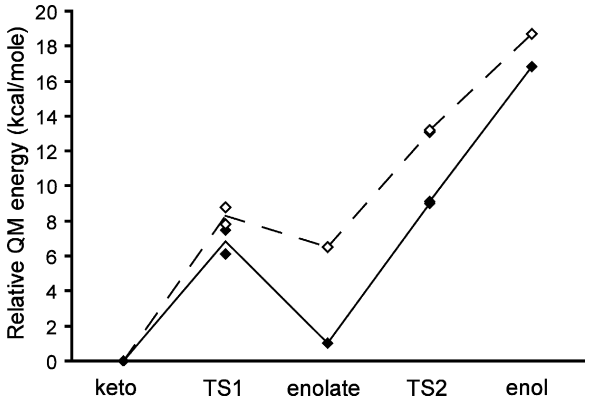


Fig. 3. MP2/6-31+G(*d*) energies for the QM system, showing the energy relative to the keto form for all QM atoms ('Asp375, His274, acetyl-CoA and Wat585', solid line) and with Wat585 deleted (dashed line).

contribution (11.6 kcal/mol) than does Wat585 (5.9 kcal/mol). The individual contributions are calculated by comparing the energies of the keto and enolate forms in the presence and absence of the hydrogen bond donor (His274 or Wat585) in question only; i.e. with the other hydrogen bonded group removed. Cooperative effects mean that the sum of these apparent contributions is not equal to the total stabilization due to both groups found by deleting them simultaneously. Similar contributions to enolate stabilization are found by comparison of the energies resulting from deletion of only one group. These values are likely to be reasonably accurate: it has been found previously that MP2/6-31+G(*d*) and RHF/6-31+G(*d*) interaction energies are close to experimental values for hydrogen bonded complexes and bracket them, with the RHF/6-31+G(*d*) results too small and the MP2/6-31+G(*d*) values slightly too large [57].

The stabilization provided by these hydrogen bonds is significant, but is less than that calculated for a bimolecular complex alone (e.g. the interaction energy of the enolate with methylimidazole is 26.6 kcal/mol (MP2/6-31+G(*d*)//6-31+G(*d*) [43])), because of interactions with the other component of the reacting system (Asp375). Wat585 and His274 donate hydrogen bonds to the carbonyl oxygen of acetyl-CoA in the substrate (keto) complex, which are stronger with the charged enolate. This increase in hydrogen bond strength upon deprotonation of the substrate stabilizes the intermediate. The energies calculated here are of course only gas-phase interaction energies, and comparison with experimental results for mutant enzymes would require free energies to be evaluated (including the effects of dielectric screening). However, it is likely that these hydrogen bonds are important in the enzyme for the stabilization of the enolate intermediate. In a low dielectric environment (such as the buried active site of citrate synthase), hydrogen bonds can undergo a large increase in strength when the acceptor atom becomes charged [58]. Such hydrogen bonds can therefore be an effective way of differentially stabilizing [59,60] the intermediate relative to the bound substrate. The hydrogen bonds in this case are of the conventional type, but both are calculated to provide considerable stabilization. The contribution of His274 (stabilizing the intermediate by 11.6 kcal/mol relative to the substrate) in particular is large. This shows that the hydrogen bond with this residue does not need to be of the low-barrier variety to stabilize the enolate significantly.

Table 3 also shows the energies along the enolization pathway calculated previously by the AM1 method for this model of the reaction (containing methylthioacetate, acetate and methylimidazole only) [43]. The geometries of all the stationary points along the reaction pathway were optimized for this model, by standard purely QM techniques. The results can be compared with the present single point calculations in which the same atoms are included (i.e. with Wat585 deleted, Table 3), although it should be remembered that these geometries will be somewhat different to those which would result from optimization without the water molecule present (e.g. the hydrogen bond between the groups representing His274 and acetyl-CoA enolate would be expected to be somewhat shorter if optimized without the water present, as noted above). Also the TS models in the present case are only approximate, unlike the exact TS found in the AM1 calculations. The relative energies of the keto, enolate and enol forms given by AM1 are very close to the MP2/6-31G(*d*) results, which is encouraging for the usefulness of the semiempirical method. Compared to the best (MP2/6-31+G(*d*)) ab initio results, however, AM1 is seen to overestimate the stability of the enol and enolate forms relative to the keto (by 6.2 kcal/mol and 4.1 kcal/mol, respectively). The AM1 barriers to proton transfer appear to be too high, but lie between the RHF and MP2 results with both basis sets, indicating that they are not unreasonable. The apparently acceptable treatment of this system by AM1, however, arises at least partially from a fortuitous cancellation of errors: AM1 underestimates the strength of the hydrogen bond between the imidazole and the enolate and overestimates the proton affinity of acetate by a similar degree, which results in the net energy change for the keto–enolate reaction being reasonably accurate [43].

For reference, Table 3 also includes the energies of the QM atoms in the various complexes produced by AM1/CHARMM calculations of the energy profile for acetyl-CoA enolization in citrate synthase. These earlier calculations used a different, slightly larger, QM system (e.g. acetyl-CoA represented by ethylthioacetate rather than methylthioacetate), and the resulting energy profile is somewhat different to AM1 findings for the smaller system. The overall pattern of energies is the same, although in the larger system the enolate complex is relatively slightly more stable, and the barrier to the keto–enolate reaction lower. AM1 gives an erroneously small energy for the hydrogen bond between His274 and the acetyl-CoA enolate [20], and consequently underestimates its contribution to stabilizing the intermediate (Table 4). The contribution of the hydrogen bond with Wat585 (5.4 kcal/mol, Table 4) calculated treating acetyl-CoA by AM1 and the water by MM (the TIP3P water model [14,61]) is more accurate [20]. It is not possible to compare the present ab initio QM/MM energies directly with those calculated earlier at the semiempirical level because the number of atoms treated QM was different. The relative total energies of the complexes along the reaction path calculated by the two methods should be comparable, however, to the extent that purely MM interactions are consistent with the QM/MM interactions. The total energies of the enolate (4.2 kcal/mol total energy) and enol (22.5 kcal/mol total energy) relative to the keto form are considerably lower than the values calculated here at the RHF/6-31G(*d*) QM/MM level (enolate 15.9 kcal/mol and enol 30.2 kcal/mol above the keto form). Treating Wat585 as MM (rather than the QM treatment in the present work) gave similar relative CHARMM energies (enolate 15.7 kcal/mol and enol 30.3 kcal/mol above the keto form), which indicates that the discrepancies between the ab initio and semiempirical results do not arise from the QM treatment of the water molecule. The differences are similar to those between the AM1 and RHF/6-31G(*d*) energies for the QM atoms alone from the respective QM/MM calculations (Table 3), and so may be deduced to arise mostly from the different levels of QM treatment, rather than from large differences in the QM/MM

interactions. AM1 calculates the enolate and enol forms to be too stable compared to the best ab initio findings, and probably overestimates the barriers to both proton transfers somewhat. The overall treatment of the energetics of this system by AM1 is qualitatively correct, however, and so the present work supports the conclusions drawn from the earlier AM1/CHARMM calculations on citrate synthase [20].

4. Discussion

Understanding the catalytic mechanism of an enzyme requires the identification of intermediates in the reaction, and the determination of how they are formed and stabilized. In citrate synthase, debate has centered on the identity of the nucleophilic intermediate, which is believed to be formed by deprotonation of acetyl-CoA. This deprotonation is probably rate-limiting [22,62], and is not concerted with the subsequent nucleophilic attack on oxaloacetate, based on experimental results for the similar malate synthase reaction [24], and for a substrate analog in citrate synthase [23]; model calculations also show a barrier to the condensation reaction [6]. The puzzle has then been how the enzyme is able to rapidly deprotonate a weakly acidic thioester using only amino acid sidechains. Pig citrate synthase has a k_{cat} of 96 s^{-1} [27] and so an apparent activation energy, $\Delta G^{\ddagger}$, of 14.7 kcal/mol (from transition state theory, $k_{cat} = \kappa(kT/h)\exp(-\Delta G^{\ddagger}/RT)$). However, the difference between the pK_a of the weakly acidic acetyl-CoA substrate (pK_a 20.4–21.5 for a simple thioester [63]) and the proposed general base, Asp375 (pK_a 6.5 [64]) implies a free energy for formation of the enolate (deprotonation of acetyl-CoA by Asp375) of $\Delta G^{\circ} = 20$ kcal/mol. Therefore, the enolate can only be an intermediate in the reaction if it is significantly stabilized by the enzyme. Based on similar considerations, it was suggested that a concerted mechanism avoiding the formation of the enolate was more probable for citrate synthase [36]. Given that the condensation with oxaloacetate is likely to be a separate reaction step, it was proposed that the enol of acetyl-CoA was formed by concerted acid–base catalysis, with His274 identified as the general acid by crystallography and mutagenesis [27,28]. In this mechanism, protonation of the acetyl oxygen by His274 is concerted with deprotonation of acetyl-CoA by Asp375, producing the enol which would then be the nucleophile for attack on oxaloacetate. The thermodynamic rationale for this proposal was undermined by the finding that the hydrogen bonding environment of His274 indicates that this sidechain is neutral (singly protonated on ND1) [20,33], and is therefore a weak acid (pK_a 14 [53]). Similarly it was demonstrated that an analogous catalytic histidine sidechain in triosephosphate isomerase is neutral over the pH range of activity of the enzyme [53,65]. With His274 neutral, formation of the enol offers no energetic advantage over the enolate, again based on pK_a's in solution ($\Delta G^{\circ} > 20$ kcal/mol [20]), and so the requirement for stabilization of the intermediate by the enzyme remains.

Similar problems of instability of reaction intermediates exist for a variety of enzymes, and it was suggested that the necessary stabilization in general is provided by a hydrogen bond between a charged intermediate and a neutral general acid at the active site (His274 in citrate synthase). The hydrogen bond was proposed to take on the special character of a low-barrier hydrogen bond in the intermediate complex, that is to say the hydrogen bonded proton would become effectively shared between the bonded partners due to a small or non-existent barrier to proton transfer. According to this proposal [35,37], such a bond is expected to be of considerably higher energy than a conventional hydrogen bond, and therefore to stabilize the high energy intermediate. In citrate synthase, this would result in His274 and an enolic acetyl-CoA intermediate sharing the ND1 proton of the imidazole sidechain. This proposal has stirred considerable debate and disagreement about its relevance to enzyme catalysis [34,38,39,41,66–68].

The metabolic importance of citrate synthase has led to the characterization of the enzyme from many organisms by a wide variety of biochemical techniques [21,22,32]. The availability of these structural and kinetic data makes the enzyme an attractive system to study by simulation techniques. A first stage in these investigations is the examination of the potential energy surface for the reaction, and specifically the stability of the species postulated as intermediates. We have examined this question using the modified structure of an inhibitor complex [29] as a representative configuration, using both standard QM and combined QM/MM techniques [5,6,20,43]. Ab initio calculations on proton transfer in bimolecular complexes stress the necessity for stabilization of any intermediate. The enolate/acetic acid complex produced by removal of a proton from methylthioacetate (representing acetyl-CoA) by acetate (representing Asp375) is highly unstable with respect to the reactants in the absence of stabilizing effects, and inclusion of electron correlation or zero-point energy removes the barrier to the reverse reaction. The enolate must be stabilized if it is to have sufficient lifetime to participate in the reaction [43]. This stabilization should be greater than that afforded in aqueous solution [36]. Similarly it is highly unfavorable to transfer a proton from methylimidazole (representing His274) to the thioester enolate in their hydrogen bonded complex [43].

The requirements for formation of a low-barrier hydrogen bond are that it should be isolated from bulk solution, the system should be charged, and that the pK_a's of the bonded partners should be approximately equal. This approximate equalization of the pK_a's should be an important signature for the presence of a low-barrier hydrogen bond. This would mean that for citrate synthase the difference between the energies of the enolate and enol complex would be expected to be significantly smaller (i.e. the deprotonation energies of His274 and the enol to be more closely matched) when the effects of the protein environment are included. These effects are taken into account approximately by the QM/MM approach, and so it would be expected that if a low-barrier hydrogen bond exists, a significant reduction in the energy difference between the enolate and enol forms would be seen on going from a purely QM treatment to inclusion of the surrounding protein at the QM/MM level. Although the results here do indicate that His274 interacts strongly with Ser244 via a hydrogen bond between the two sidechains, this does not greatly reduce the

energy difference between the enolate and enol, which remains large. These findings show no indication of pK_a equalization of the intermediate and the electrophilic catalyst by the enzyme. Alternatively it might be suggested that the proton-sharing structure of the low-barrier hydrogen bond, with an associated reduced separation between the heavy atoms, should be studied to assess its energy, and that it is important to include electron correlation to describe such a species correctly. This has been addressed here by modelling such structures with the hydrogen bonded proton equidistant between the enolate oxygen of acetyl-CoA and ND1 of His274. With or without MP2 correlation corrections applied to the QM system in the 6-31G(*d*) QM/MM calculations, the enolate is significantly more stable than these models (by 7.5 kcal/mol when MP2/6-31G(*d*) corrections are added to the 6-31G(*d*) QM/MM energies). The results therefore provide no support for the hypothesis that a low-barrier hydrogen bond with His274 is responsible for stabilizing the reaction intermediate. In any strongly hydrogen bonded system, some degree of proton transfer can be expected to occur, and a full description would require the quantum dynamics of the system to be taken into account. The present calculations indicate, however, that the conventionally hydrogen bonded structure is the most stable, and so the best description of the intermediate in citrate synthase is the enolate of acetyl-CoA, stabilized by a normal hydrogen bond from His274 (and from an active site water molecule). These hydrogen bonds stabilize the enolate relative to bound substrate, and may prevent its decomposition. The enolate character of the intermediate is likely to be important for the condensation with oxaloacetate to proceed efficiently [6].

Proton abstraction from acetyl-CoA by Asp375 in citrate synthase and the possible enolate, enolic and enol intermediates formed thereafter have also been studied by Yang and Drueckhammer [69] using a two layer ONIOM approach [70]. Ab initio QM methods (HF/6-31+G* for geometries and MP2 and B3LYP energy calculations with the same basis set) were used on a reaction region identical to the QM treated part in this study, and PM3 was used on a 10 Å radius region around the terminal acetyl-CoA carbon. Their results further confirm that the enolate is the true intermediate, with the enolic and enol intermediates much higher in energy. These authors found, as here, a large effect of correlation for the proton abstraction: including MP2 corrections the energy barrier is much lower than at the HF level. The barrier heights they find are similar to values we report (22.9 kcal/mol versus 21.8–22.0 kcal/mol for HF/6-31+G(*d*), 12.7 kcal/mol versus 14.6–13.8 kcal/mol for MP2/6-31+G(*d*)). Differences between these values can be attributed to the differences in the approaches used. Although the exact geometries differ slightly (due to different setup of the models, etc.), the hydrogen bond distances to the acetyl-CoA thioester oxygen donated by His274 and the conserved water molecule follow the same trend along the proton transfer: the distances shorten going from the keto to the enolate intermediate, in response to the developing negative charge on the oxygen.

If proton abstraction from acetyl-CoA is indeed the rate-limiting step in citrate synthase, its free energy barrier is about 14.7 kcal/mol (calculated from the experimentally determined reaction rate, see above). The QM/MM barrier we find for this proton abstraction with the highest QM level (HF/6-31G/CHARMM total energy with MP2/6-31+G(*d*) correction) is 14.6–13.8 kcal/mol and is therefore consistent with the experimental value. The equivalent energies for the 'enolic' and enol species as reported here (22.4 kcal/mol and 27.6 kcal/mol, respectively), are not consistent with experiment, again indicating that the enolate is the true intermediate. The close agreement between our QM/MM barrier and the experimental barrier is probably fortuitous, as several theoretical and methodological effects will cause a difference between the two. Most importantly, the barrier obtained from the experimental reaction rate is a free energy difference, whereas our calculated energy difference, based on QM/MM energy minimization, does not include entropic effects (and is therefore better compared to a reaction enthalpy). Further, two effects can cause underestimation of the barrier in our approach: (1) we calculate an approximate transition state, with the proton equidistant from the acetyl-CoA carbon and the Asp375 oxygen, whereas the 'real' transition state is likely to be further along the reaction coordinate ([69]; van der Kamp and Mulholland, unpublished results) and somewhat higher in energy, and (2) shortcomings in the MP2 method [71] probably cause underestimation (van der Kamp and Mulholland, unpublished results), as is the case for reactions barriers in chorismate mutase and *para*-hydroxybenzoate hydroxylase [18]. On the other hand, quantum effects such as tunnelling [72,73] and zero-point energy (ZPE) can in turn reduce the reported barrier somewhat. The effect of tunnelling is expected to be small, however, because of the large difference in energy between keto and enolate and the small kinetic isotope effect in citrate synthase [62,74]. The ZPE effect on the barrier can be estimated to reduce the barrier with about 3 kcal/mol, based on small model calculations [43].

Our conclusion that catalysis in citrate synthase does not involve low-barrier hydrogen bonds agrees with calculations on TIM, in which the analogous catalytic His95 has been found to have no tendency to donate a proton to the enediolate intermediate, and instead to stabilize it by a normal hydrogen bond [38,75]. Experimental results show that while unusually short hydrogen bonds can be formed in enzymes, including citrate synthase, they do not lead to exceptionally large binding energies [76,77]. The strongest, shortest hydrogen bonds are found for charged interactions between groups with approximately equal pK_a's [51,78], but there is no special stabilization associated with disappearance of the barrier to proton transfer [42], or at ΔpK_a 0 [79]. Theoretical analysis indicates that low-barrier hydrogen bonds do not offer an energetic advantage for enzyme catalysis [40,41]. Low-barrier hydrogen bonds may form in some proteins [34], but it is not necessary for hydrogen bonds to be of this type to stabilize high energy (unstable) intermediates. Stabilization of the enolate intermediate in citrate synthase is achieved by hydrogen bonding, but not low-barrier hydrogen bonding.

The geometries produced by ab initio QM/MM energy minimization of the enzyme complex are well minimized, as

shown by comparison to fully optimized bimolecular complexes. This shows that the ab initio QM/MM method can be a useful tool to optimize the geometries of substrates and intermediates bound at enzyme active sites, treating the QM system accurately while including the bonded and non-bonded restraints due the surrounding enzyme, which are essential in a full description. This is a good way to study the structures and energies of important species in a reaction, but the computational demands of ab initio QM/MM calculations are often too high to perform dynamics simulations of reacting enzyme complexes from which free energy profiles of enzymatic reactions can be obtained. Such simulations require simpler QM treatments, for example at the semiempirical molecular orbital [14] or empirical valence bond level [80]. Ab initio QM/MM calculations should be an effective way of testing these methods. Alternatively, free energy of reactions can be modelled using the QM-FE [81] or QM/MM-FE [82] approaches using ab initio level quantum mechanics. Donini et al. [83] applied the QM-FE approach to proton abstraction in citrate synthase. In this approach, the QM region (Donini et al. used a smaller region than used here, consisting of the sidechains of Asp375 and His274 and methylthioacetate) in the QM-FE method is optimized in the gas phase (level: HF/6-31+G(*d*)) with several constraints to keep it in an enzyme-like conformation. In the subsequent MD simulation of the solvated enzyme–substrate system, restraints are used to keep the geometry close to the QM determined one. In our QM/MM simulations, there is no restriction on the movement of the QM atoms apart from the position of the link atoms attached to the MM enzyme environment. There are also essential differences in the model setup between this work and Donini et al. The most important of these is a consequence of the choice of Donini et al. to base their model on a crystal structure without water molecules (PDB-code 2CTS [84]). After their solvation procedure, they find a water to be interacting with both Asp375 carboxylate oxygens in the keto-form, and to be bridging between Asp375 and the carbon from which a proton is abstracted in the enolate form. These structural features are not seen in our study nor in the work of Yang and Drueckhammer [69]. Despite these differences, Donini et al. also found that an enol intermediate would be much less stable than the enolate intermediate ($\Delta\Delta G$ of 13.6 kcal/mol, which compares well to the total QM/MM energy difference including MP2 correction we find of 13.1 kcal/mol.). Donini et al. further reported a total free energy difference between the keto and the enolate form of 15.4 kcal/mol which consists of a MP2/6-31+G(*d*) (potential) energy difference between keto and enolate of 18.4 kcal/mol and a 'free energy component' of −3.0 kcal/mol. The MP2/6-31+G(*d*) energy difference can be compared to the energy of 'Asp375, His274 and acetyl-CoA only' we report in Table 3 of 6.5 kcal/mol. The significant difference between these values can be due to the limited optimization of the QM region in the QM-FE approach as described above. This indicates that for optimizing geometries of enzyme reactions, it is crucial to take the enzyme environment into account, for example using our QM/MM approach. Overall, though, it is encouraging that all the calculations on citrate synthase, using different theoretical approaches, agree qualitatively: all identify the same probable mechanism, with the acetyl-CoA enolate as the nucleophile.

It is important to bear in mind the well-known inadequacies of ab initio calculations at the Hartree-Fock level for problems of chemical reactivity. In many cases, (hybrid) DFT methods like B3LYP will be a better option [15,85]. For the proton abstraction from acetyl-CoA in citrate synthase, however, this is not the case: B3LYP significantly overestimates the reaction energy (van der Kamp, Mulholland, unpublished results). When QM/MM calculations are limited to the HF level of QM treatment, it is important to consider the effects of electron correlation. This point is underlined by the findings of the present study that correlation corrections (applied at the MP2 level to the QM system) significantly affect the reaction energetics. Similarly, small basis sets can give misleading results. Semiempirical methods can be preferable to low-level ab initio treatments, as the semiempirical results can in some cases be closer to those from correlated ab initio calculations and of course can be performed much more quickly. The semiempirical parameters can be optimized or corrected for a particular application to fit ab initio or experimental results for a reaction, an effective approach for QM/MM calculations [86,87]. More recent developments such as the self-consistent charge density-functional tight-binding method (SCC-DFTB) [88], an approximate density-functional approach, are also promising in this respect. The MM parameters used to represent van der Waals interactions between the QM and MM system should ideally also be optimized to reproduce interaction energies and geometries calculated at the ab initio level for detailed analysis of QM/MM interactions and free energy changes [14,87,89,90]. Given such flexibility and computational efficiency, combined with testing against ab initio QM/MM results, the prospect of semiempirical QM/MM simulations providing useful insight into enzyme reaction dynamics is still bright.

5. Conclusions

Ab initio QM/MM calculations are a useful way to model enzyme reactions, including the effects of the enzyme and other groups on the reacting system. The present study has shown that the enolate of acetyl-CoA is the likely nucleophilic intermediate in citrate synthase, stabilized by hydrogen bonds from His274 and a water molecule. The results provide no support for the hypothesis that the hydrogen bond between His274 and the intermediate is of the 'low-barrier' type, and show that normal hydrogen bonds provide significant stabilization of the intermediate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmgm.2007.04.002.

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