Ab Initio QM/MM Study of the Citrate Synthase Mechanism. A Low-Barrier Hydrogen Bond Is not Involved

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Quantum mechanical/molecular mechanical (QM/MM) methods offer an approach to understanding enzyme reaction mechanisms at the atomic level.[1] Although calculations on enzymes at semiempirical QM levels[2] have led to valuable insights,[3−6] more accurate ab initio QM methods[7] are required to obtain reliable results for some systems. One such case is the reaction catalyzed by the enzyme citrate synthase (CS). Use of an ab initio approach, including the effects of the enzyme environment, is particularly important for this reaction because of the possible role of so-called "low-barrier" hydrogen bonds (LBHB).[8,9] To address their importance, the correct relative energies of different postulated intermediate forms (Figure 1) are required. Results with an ab initio QM/MM method,[7] identify the enolate of acetyl-CoA as the nucleophilic intermediate. They show that it is stabilized by normal (not low-barrier) hydrogen bonds with a conserved histidine residue and a water molecule.

CS catalyzes the metabolically important formation of citrate from acetyl-CoA and oxaloacetate. Asp-375 (numbering for pig CS) has been shown to be the base for the rate-limiting deprotonation of acetyl-CoA[10] (Figure 1). An intermediate (which subsequently attacks the second substrate, oxaloacetate) is believed to be formed in this step and is stabilized by a hydrogen bond with His-274.[4,10b] It is uncertain from the experimental data whether this intermediate is the enolate or enol of acetyl-CoA; related questions arise in similar enzymatic reactions.[1,5,11] From the relative pK_as of Asp-375 and acetyl-CoA, it appears that the enolate (or enol) can only be an intermediate in the enzymatic reaction if it is stabilized by the enzyme.[4,7] It has been proposed that the necessary stabilization is provided by a LBHB.[8] The suggested LBHB is between His-274, which is neutral, and an "enolic" acetyl-CoA intermediate.

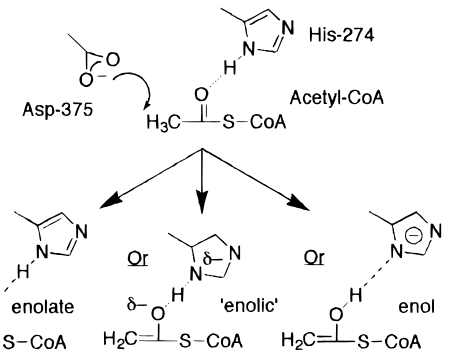

Figure 1. Mechanism of citrate synthase, indicating the possible alternative enolate, enol, and enolic intermediate forms of acetyl-CoA.

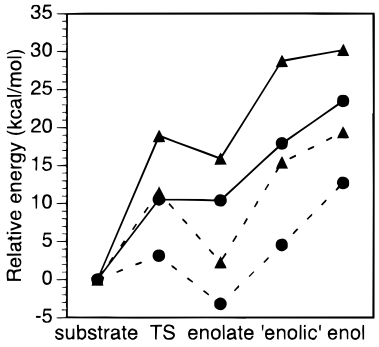

Figure 2. Energy profile for enolization of acetyl-CoA in citrate synthase: solid lines show QM/MM energies for the reaction in the enzyme (▲, RHF/6-31G(*d*); ●, MP2/6-31G(*d*)). The dotted lines shows the energy of the QM system alone. Energies are given in kcal/mol relative to substrate. For the TS for proton abstraction from acetyl-CoA by Asp-375, and for the enolic intermediate, the mean energy of two (very similar) results is shown.[24]

To resolve the question of the nature of the intermediate and its stabilization by the enzyme, we have investigated the first reaction step in CS by ab initio QM/MM calculations with the CHARMM program.[12] The simulation system contained all residues within 17 Å of the terminal carbon of acetyl-CoA in a high-resolution crystal structure of chicken CS with the inhibitor *R*-malate.[13]

The calculated energy profiles for enolization of acetyl-CoA in CS are shown in Figure 2. Within the enzyme, the substrate (keto form of acetyl-CoA) has the lowest energy. Of the postulated intermediates, the enolate of acetyl-CoA is more stable than the enol or enolic forms. The relative energies of the different forms are altered by inclusion of electron correlation, but the results are qualitatively similar at the RHF and MP2 levels. The enolate lies 10.4 kcal/mol higher than the substrate complex, whereas the enol and enolic forms have energies of 23.9 and 17.9 kcal/mol relative to the substrate complex (MP2/6-31G(*d*) QM/MM). The calculated energy change for formation of the enolate is consistent with the experimentally estimated activation energy (14.7 kcal/mol[4]). The effects of the surrounding protein and the dianionic second substrate, oxaloacetate, on the reacting system can be obtained by comparing the QM/MM energies with the energy calculated for the QM atoms alone (Figure 2). Many

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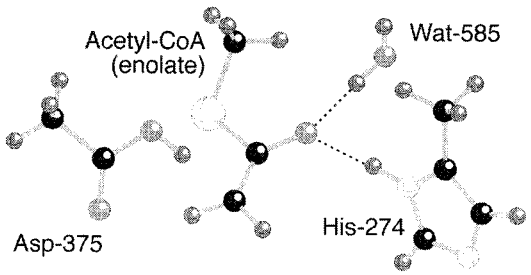

Figure 3. Geometry of the acetyl-CoA enolate bound to citrate synthase produced by RHF/6-31G(*d*) QM/MM energy minimization, showing the QM atoms only.

groups contribute significantly to the overall reaction energetics and that there are significant indirect effects: e.g., there is a strong interaction between Ser-244 (a conserved residue[4]) and Nϵ2 of His-274 (the Nδ1 of which donates an essential hydrogen bond to the substrate, see below). The problem faced by citrate synthase in deprotonating acetyl-CoA is 2-fold. Not only does the enzyme face the difficulty of deprotonating a weakly acidic carbon acid substrate and stabilizing the resulting enolate (a problem that is apparent from a comparison of the pK_as of the base and acetyl-CoA), but it must do so with oxaloacetate bound in close proximity. Oxaloacetate is the second substrate and reacts with the enolate in the second reaction step, but because it is a dianion, its interaction with the negatively charged enolate is highly unfavorable. The enolate is strongly destabilized by oxaloacetate (relative to acetyl-CoA), but they are bound close together. The enolate acts as a nucleophile to attack the carbonyl carbon of oxaloacetate to form a citryl-CoA intermediate, en route to formation of citrate.

His-274 and Wat-585 are the groups primarily responsible for stabilizing the enolate intermediate (both were included in the QM system). They do so by donating hydrogen bonds to the carbonyl oxygen of acetyl-CoA (Figure 3). These bonds exist in the substrate complex, but are strengthened upon deprotonation of acetyl-CoA. The contributions of His-274 and Wat-585 can be estimated by comparing the energies of the enolate and substrate forms of acetyl-CoA with and without these hydrogen bonding groups present. The stabilization of the enolate relative to the substrate due to His-274 is 13.1 kcal/mol, and that due to Wat-585 is 6.5 kcal/mol; the stabilization with both groups present is 18.8 kcal/mol due to small nonadditive effects. This stabilization of the enolate in citrate synthase occurs through "normal" hydrogen bonds in which the protons are covalently bonded to their respective donor atoms. In the overall energy profile (Figure 2), this stabilization is offset by unavoidable destabilization of the enolate by oxaloacetate as described above.

If a LBHB were important in catalysis of deprotonation of acetyl-CoA by citrate synthase, the enolic intermediate would be expected to be lower in energy than (or at least comparable to) other possible intermediate forms. This is not found in the present calculations, which go beyond a Hartree−Fock treatment to ensure that such partially bonded species are represented at the required level of accuracy. LBHBs have been suggested to be very strong and provide stabilization much greater than a normal, charged hydrogen bond.[8] However, the model of the enolic intermediate which represents the LBHB species (and in which the hydrogen-bonded proton is approximately equidistant between Nδ1 of His-274 and the enolate oxygen, and the N···O distance is 2.48 Å, corresponding to that in very short hydrogen bonds) is significantly higher in energy than the normal hydrogen-bonded enolate (e.g. by 7.5 kcal/mol, MP2/6-31G(*d*) QM/MM). This is true in the presence or absence of Wat-585, and whether or not the effects of the protein environment are included.

Another indicator of the possibility of a LBHB relates to the criterion[8] that the effective pK_a of the intermediate should be approximately equal to that for deprotonation of neutral His-274 for a LBHB to form (i.e., the proton affinities of the hydrogen-bonded imidazolate and enolate should be very similar, so that the energetic penalty for proton transfer is small). This would require the energies of the enolate and enol to be similar at the active site. This is not the case (the energy difference between the enolate and enol forms is 13.1 kcal/mol (MP2/6-31G(*d*) QM/MM) when the effects of the MM enzyme environment are included, and 15.9 kcal/mol for the QM atoms). Thus the conditions for formation of a LBHB are not satisfied. The same conclusion is obtained at the RHF and MP2 levels, with or without Wat-585 present. That the enolate is the probable reaction intermediate is in accord with lower level QM/MM calculations.[1,4] The reaction energetics vary somewhat depending on the MM charges employed to represent the surrounding groups, but at all levels and under all conditions tested the enolate form is calculated to be markedly lower in energy than the enolic or enol forms of acetyl-CoA.

A primary goal in the investigation of an enzyme-catalyzed reaction is the identification of intermediates in the reaction, and an understanding of how they are formed and stabilized. The present results, identify the enolate of acetyl-CoA as the intermediate formed by the first step of the citrate synthase reaction. The intrinsic instability of the thioester enolate is overcome by normal hydrogen bonds with His-274 and an ordered water molecule (Wat-585). In the buried active site, these hydrogen bonds with the charged enolate are considerably stronger than those with the neutral substrate carbonyl group,[14] but they do not satisfy the conditions for a LBHB. They differentially stabilize[15] the enolate, which is a good nucleophile for attack on oxaloacetate in the next step of the reaction,[16] and allow the overall reaction forming citrate to proceed efficiently.

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Supporting Information Available: A description of the calculational procedures is given (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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