

# DFT/PM3 study of the enoyl-CoA hydratase catalyzed reaction

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**Abstract** The enoyl-CoA hydratase catalyzed hydration of  $\alpha,\beta$ -unsaturated thioesters has been modeled by using the crystal structure of 4-(*N,N*-dimethylamino)cinnamoyl-CoA bound at the active site. The quantum chemical calculation used the ONIOM mixed level procedure to permit the substrate thioester and water molecule to be modeled using B3LYP/6-31G(d) level of theory and the active site residues modeled at a semiempirical level using the PM3 Hamiltonian. The results permitted the identification of a stable thioester enolate intermediate, supporting a stepwise reaction mechanism. The calculation also suggests that the same proton removed from the nucleophilic water molecule is transferred to  $C\alpha$  in the subsequent protonation of the enolate intermediate. This observation reconciles the stepwise mechanism with the previously reported double isotope effect study [3].

**Key words** coenzyme-A • density functional theory • enoyl-CoA hydratase • kinetic isotope effect • mechanism • ONIOM • thioester enolate

## Introduction

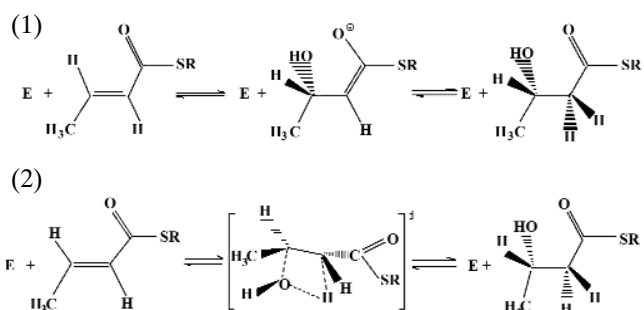
Enoyl-CoA hydratase (ECH) catalyzes the syn addition of the elements of water across the double bond of  $\alpha,\beta$ -unsaturated CoA thioesters. This reaction has become the paradigm for the isomerase/hydratase superfamily [11, 13]. This superfamily is characterized by a binding site for CoA thioesters that polarizes the carbonyl by two amide H-bonds, one to a glycine at the N-terminus of a conserved  $\alpha$ -helix and one to a second amide, Ala-98 in ECH [7].

Initial isotope effect studies revealed that there were significant primary  $^{18}\text{(V/K)}$  and primary  $^{\text{D}}\text{(V/K)}$  kinetic isotope effects (KIEs) on the dehydration reaction [2]. These isotope effects suggested two possibilities: a step-wise reaction with the  $C\alpha$ -H bond being cleaved to enerate a stabilized thioester enolate and subsequent cleavage of the  $C\beta$ -O bond to generate the product  $\alpha,\beta$ -unsaturated product where both transition states are partially rate determining (Eq. (1)); alternatively the reaction could be concerted with both the  $C\alpha$ -H and  $C\beta$ -O bonds being cleaved in a single transition state.

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A double isotope effect study [10] was performed to discriminate between these two possibilities. The effect of  $^2\text{H}$  on the  $\alpha$ -secondary  $^2\text{H}$  KIE was determined to be negligible leading to the conclusion that the reaction was concerted [3]. The reaction was proposed to be concerted because the intermediate enolate would not have a significant lifetime. However others have suggested that the reaction should be stepwise [9] and other reactions of this enzyme class have been shown to have enolate intermediates [12, 15].

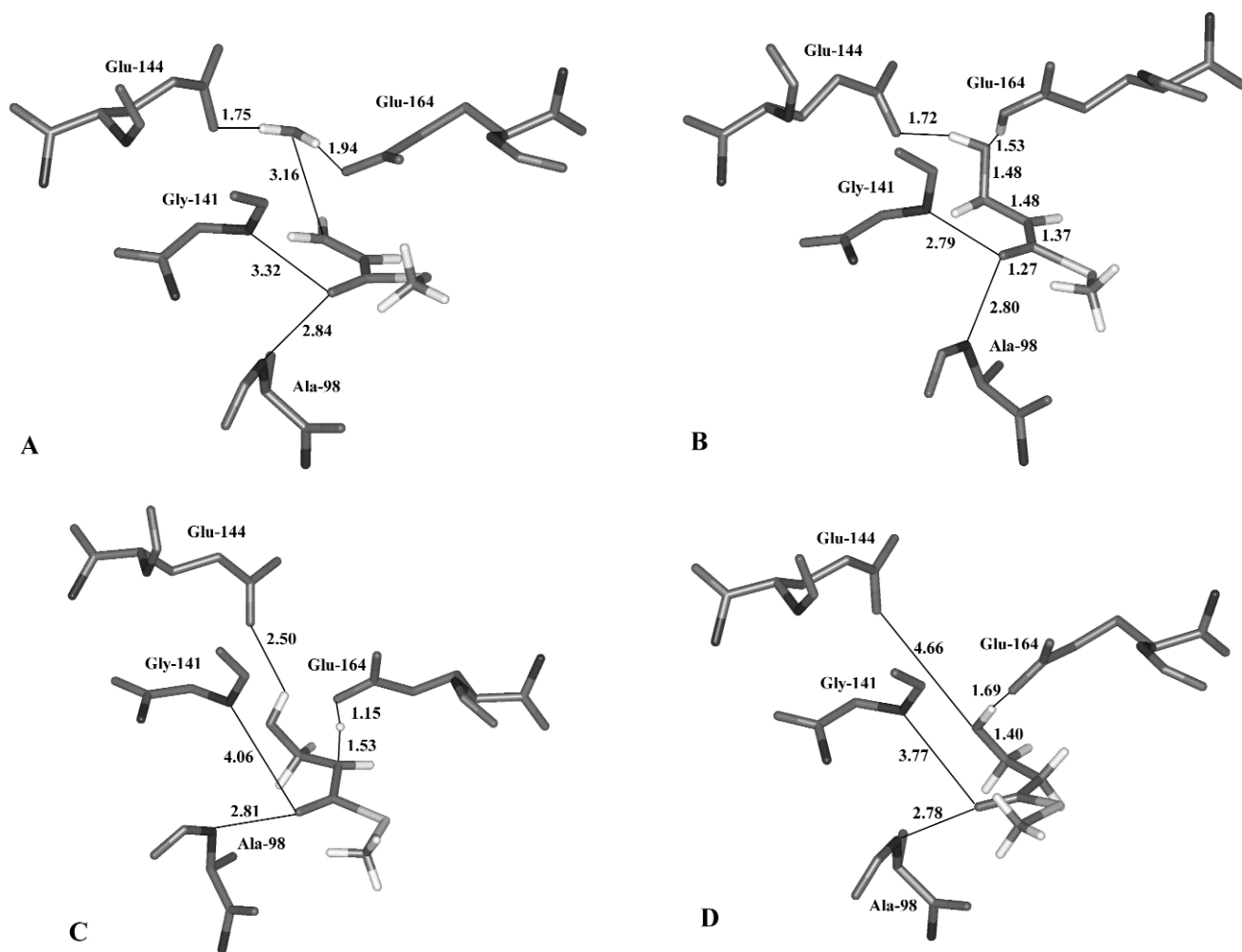
Following the determination of the crystal structure ECH with 4-(*N,N*-dimethylamino) cinnamoyl-CoA (DAC-CoA) bound at the active site, we have been pursuing an ONIOM(DFT/PM3) model of the reaction. These studies have revealed that an enolate intermediate is stabilized in the active site and provide an alternative explanation for the previously reported double isotope effects [3].

## Methods

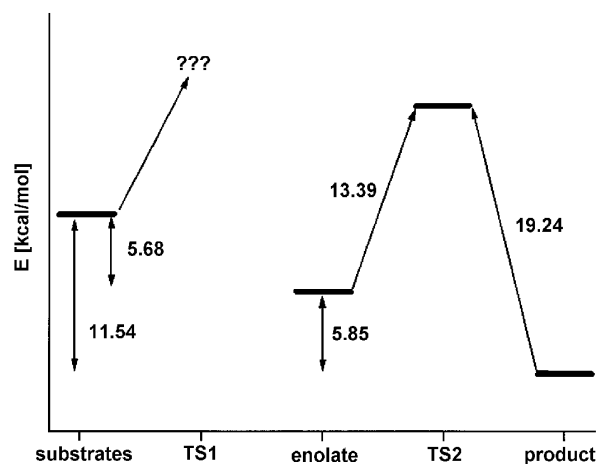
The crystal structure of DAC-CoA bound to ECH determined by Bahnson *et al.* [3] (1EY3) was used as the starting structure for these computational studies. This structure is the highest resolution ECH structure available and impor-

tantly, has identifiable electron density for the substrate water molecule located, and by implication, H-bonded between the two active site carboxylates of Glu-144 and Glu-164 that function as general acid/base groups [6, 14]. To construct the ONIOM model [4] of the enzyme reaction, the DAC-CoA was truncated to the *S*-methyl propenoyl thiolester whose CoA analog is known to be a substrate. All active site residues within 5 Å of the  $\text{C}\alpha=\text{C}\beta$  double bond were then selected and included in the model. The protein backbone was capped on the amino ends as amides of formic acid [ $\text{H}(\text{C}=\text{O})\text{-NHR}$ ] and on the carboxylate ends as amides [ $\text{R}(\text{C}=\text{O})\text{-NH}_2$ ]. The atoms of the propenoyl-thiolester, the substrate water molecule and Glu-164 were included in the high level DFT region and the rest of the active site atoms assigned to the PM3 region. The backbone atoms of the protein were restrained. The optimization proceeded until the standard Gaussian convergence criteria for both maximum and RMS forces were met, but because of the flatness of the energy surface, the standard criteria for maximum and RMS displacements were not met. Following this optimization, minimal reorganization of the active site side chains occurred.

The propenoyl thiolester was hydrated by forming a C-OH bond to  $\text{C}\beta$  and a C-H bond to  $\text{C}\alpha$  in the active site with the



**Fig. 1.** The ONIOM(DFT:PM3) model reaction pathway for the ECH catalyzed hydration of an  $\alpha,\beta$ -unsaturated thiolester is shown in four panels. Phe-263, Gly-172, Ala-173, Trp-120, Leu-117 and Met-103 have been omitted from the Figures to permit functional active site residues to be more readily visualized. Labeled distances are in Å. A – The ONIOM(DFT:PM3) optimized model of the bound  $\alpha,\beta$ -unsaturated *S*-methyl propenoyl thiolester. B – The enolate intermediate following addition of water to  $\text{C}\beta$  and proton transfer to Glu-164. The stabilizing H-bonds from Gly-141 and Ala-98 are shortened. C – The transition state for transfer of the water derived proton from Glu-164 to  $\text{C}\alpha$  is shown. D – The hydrated product, *S*-methyl-3-hydroxyl propanoate, bound at the ECH active site is shown.



**Fig. 2.** Reaction coordinate diagram for the ECH reaction determined by the ONIOM(DFT:PM3) calculation. The transition state for C $\beta$ -O bond cleavage has not yet been characterized, but the large experimental  $^{18}\text{V}/\text{K}$  indicates this is the rate determining step. The enolate intermediate is clearly stabilized with a significant barrier both to C $\beta$ -O bond cleavage and to protonation at C $\alpha$  by Glu-164. The reaction equilibrium correctly favors hydration of the propenoyl-thioester.

appropriate stereochemistry relative to the active site functional groups. This structure was optimized by examining different H-bonding pattern between the C $\beta$ -OH and both active site glutamates. The enolate intermediate was generated by transferring the proton from C $\alpha$  to Glu-164 and optimizing the resulting structure. The transition state for proton transfer was located by the QST2 procedure [1] implemented in Gaussian 98 [8]. This intermediate was judged to be stable since the optimization of forces converged (*vide supra*) and there were significant barriers to the apparent proton transfers.

## Results and discussion

The initial minimized model of the ECH active site is shown in Figure 1A. This model reproduced the observed significant perturbations in the  $^{13}\text{C}$  NMR shieldings for the carbonyl  $\alpha$ -, and  $\beta$ -carbons [5] of enoyl-CoA substrates. The second structure Figure 1B is the key enolate intermediate. The proton from the initially bound water molecule has been transferred to Glu-164 while the C $\beta$ -OH is H-bonded to Glu-144. This structure is stable in the enzyme active site, the C $\beta$ -O bond is orthogonal to the C $\alpha$  = C-O $^-$  enolate as anticipated. The thermodynamic stability of this product strongly suggests that this enzymatic reaction should be stepwise. The stability extended to an alternative structure where the proton on Glu-164 was transferred to the C $\beta$  hydroxyl group.

Figure 1C is the transition state for proton transfer from Glu-164 to C $\alpha$  of the substrate located by the QST2 procedure. Figure 1D is the model for the bound hydrated product. Following the reaction in the dehydration direction (1D  $\rightarrow$  1A) provides an explanation for the observed double isotope effects. The proton labeled to obtain the primary  $^2\text{H}$  effect is involved in two proton transfer reactions: from C $\alpha$  to Glu-164 and then from Glu-164 to the OH leaving group. The crystal structure emphasizes that there are no other sources for the proton donated by the general acid.

Thus both bond cleavage steps will be sensitive to  $^2\text{H}$  substitution at the *pro*-2. This suggests that the observed  $^{\text{D}}(\text{V}/\text{K})$  arose not from cleavage of the C $\alpha$ -D bond but from transfer of the same primary proton during the rate determining C $\beta$ -O cleavage step.

Figure 2 is an energy diagram for the four structures in Figure 1. As anticipated the hydration reaction for this substrate is favorable. The stability of the enolate intermediate is emphasized, as it is more stable than the  $\alpha,\beta$ -unsaturated substrate. Two features of the active site promote the stability of the enolate intermediate. As electron density is transferred to the carbonyl O, the two H-bonds from the amides of Gly-141 and Ala-98 are strengthened and the unfavorable interaction between the two active site negative charges is relieved. The  $\Delta\text{G}$  of proton transfer from Glu-164 to the enolate C $\alpha$  is favorable, but is much smaller than would be anticipated for groups with solution  $\text{p}K_{\text{a}}$ s of 4.7 and  $\sim 22$ , suggesting a strong stabilization of the enolate by the active site.

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