VIP Enzyme Stereospecificity

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"Lethal Synthesis" of Fluorocitrate by Citrate Synthase Explained through QM/MM Modeling**

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The term "lethal synthesis" was coined for enzymatic formation of fluorocitrate,[1] but this classic problem of enzyme stereoselectivity remains poorly understood. Here, we show that high-level ab initio quantum mechanics/molecular mechanics (QM/MM) modeling can accurately capture this enzymatic enantioselectivity and the results provide detailed insight into its origin. Citrate synthase (CS) performs the first reaction in the citric acid cycle: the formation of citrate from oxaloacetate and acetate in the form of acetyl-CoA. When fluoroacetyl-CoA (from fluoroacetate) is used as a substrate instead of acetyl-CoA, 2-fluorocitrate is formed,[2] which inhibits aconitase,[3,4] the next enzyme in the citric acid cycle. This process is responsible for the lethal toxicity of fluoroacetate to humans and other mammals.[5] The fluorocitrate enantiomer that is predominantly formed by CS, (2*R*,3*R*)-fluorocitrate (Figure 1), is the same enantiomer that specifically inhibits aconitase.[4] The only (semi-)quantitative experimental study published to date indicates that the minor product of the enzymatic formation of fluorocitrate, (2*S*,3*R*)-fluorocitrate, amounts to 2–3% of the major product.[6] Using transition-state theory, this translates to a difference in activation free energy ($\Delta\Delta G^{\ddagger}$) of 2.06–2.30 kcal mol^{-1}. The causes of this selectivity remain uncertain.

We have previously modeled the two initial reaction steps for the natural substrates, proton abstraction from acetyl-CoA and condensation with oxaloacetate (OAA), in CS with high-level QM/MM methods.[7–9] For the reaction with fluoroacetyl-CoA (FaCoA), the distinction between enantiomers is made in the proton-abstraction step, where either an *E*- or a *Z*-enolate is formed. Here, we show that 1) the calculated relative energy of the enolates accurately predicts the experimentally observed enantiospecificity and 2) the enantiospecificity is mostly due to the inherent energy difference of the reacting species.

A model of the enzyme with OAA and FaCoA bound was built for QM/MM simulation, with the Asp375 side chain from Cβ, the methylthioester part of FaCoA, and OAA in the QM region. QM/MM molecular dynamics indicated that pre-*E* and pre-*Z* conformations in the CS active site can be sampled in the same trajectory. Five different approximate transition-state conformations for both *E*-enolate and *Z*-enolate formation were generated by QM/MM umbrella sampling molecular dynamics (see the Supporting Information). These conformations were used to perform high-level QM/MM modeling of proton abstraction, using the established reaction coordinate $r = d(O_{Asp375}H) - d(C_{FaCoA}H)$.[7–9] Geometries were optimized at the B3LYP/6-31 + G(d)//MM level and energies calculated at the SCS-MP2/aug-cc-pVDZ//MM level. This describes the reaction of CS with acetyl-CoA accurately, in agreement with local coupled-cluster QM/MM results.[8]

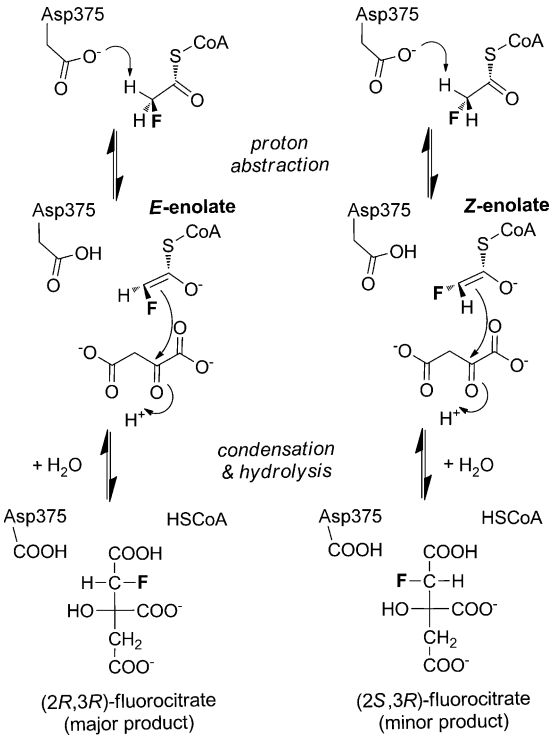

Figure 1. Conversion of fluoracetyl-CoA to fluorocitrate by CS.

The QM/MM energy profiles for enolate formation show correctly that formation of the *E*-enolate is preferred (Figure 2). Boltzmann-weighted energy differences between the *E* and *Z* profiles are 1.80 and 2.05 kcal mol^{-1} for the activation energy and reaction energy in this step, respectively. Entropy contributions for *E*- and *Z*-enolate formation are expected to be (nearly) identical (supported by AM1/MM free energy profiles, see the Supporting Information). The

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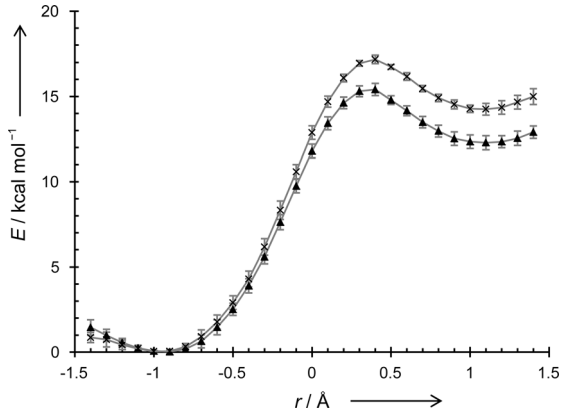

Figure 2. QM/MM (SCS-MP2/MM) energy profiles of the formation of *E*- (▲) and *Z*- (×) enolates from fluoroacetyl-CoA in CS. Average profiles (relative to the substrate complex) from five different enzyme–substrate conformations are shown, with error bars indicating standard deviations.

potential energy difference for enolate formation agrees very well with the difference deduced from experimental data (2.06–2.30 kcal mol^{-1}), indicating that differences in energy for the enzyme-bound enolates can explain the enantioselectivity of CS for FaCoA.

To investigate the reasons for this preference, the energy of the optimized gas-phase models of the *E*- and *Z*-enolates was compared to the equivalent energy from the conformations obtained from QM/MM modeling in the enzyme active site. Gas-phase models reveal a 1.70 kcal mol^{-1} difference in favor of the *E*-enolate, that is, the inherent energy difference. A similar difference (from five conformations each) is obtained for the QM/MM models: (1.62 ± 0.3) kcal mol^{-1}, indicating that the inherent difference is still present in the enzyme active site. The gas-phase energy difference is similar to previous results for small models of enols and enolates, which led to a proposal that the enol, rather than the enolate is the nucleophilic intermediate in the CS reaction.[10]

In citrate synthase, the chemical conversion is rate-limiting for product formation[11] and selectivity is therefore related to a difference in energy barriers along the reaction. Comparing relative energies (vs. the enzyme–reactant complex) of the reacting species, rather than the absolute energies of the enolates in the enzyme active site, may thus give insight into the origin of enantioselectivity (Table 1). When only FaCoA is considered, the relative activation and reaction energies between *E*- and *Z*-enolate are slightly larger than the absolute energy difference (1.6 kcal mol^{-1}, see above). This difference between the absolute and relative energy difference is due to the lower energy (0.9 kcal mol^{-1} on average) of the pre-*Z* conformation of FaCoA in the CS active site, which indicates that selectivity is not a result of a less favorable pre-*Z* reactant conformation in the enzyme. When the Asp375 side chain is included (in addition to FaCoA), energy differences are smaller than in the full QM/MM system, indicating that a direct interaction between FaCoA and the catalytic base also does not contribute to selectivity, contrary to previous suggestions.[12] The same is true when OAA is also included, to arrive at the full QM region used in this study. The latter indicates that differences in the enzyme environment are likely to contribute somewhat to enantioselectivity. This was confirmed by calculating the energy of the MM region during *E*- and *Z*-enolate formation (see the Supporting Information). The differences are subtle (Figure 3) and probably reflect unfavorable steric interactions of the fluorine in the *Z*-enolate-forming reaction. Overall, the enantioselectivity of CS towards fluoroacetate appears to arise primarily from the inherent energy difference between the *E*- and *Z*-enolates, with further small contributions from interactions in the CS active site. Formation of the stable citryl-CoA intermediate is believed to be the rate-limiting step for reaction in mesophilic CS.[11] Calculations suggest that condensation with OAA (and not proton abstraction from acetyl-CoA) may be rate limiting.[9] If the same is true for the reaction with fluoroacetyl-CoA, the energy difference that arises in enolate formation must be carried through to the highest energy barrier in the formation of fluorocitryl-CoA. Calculations on small models indicate that this is indeed the case (see the Supporting Information).

The prediction of selectivity in enzyme-catalyzed reactions has potential practical applications in catalyst design and drug metabolism.[13] It presents significant challenges owing to

Table 1: Relative energy differences between *E*- and *Z*-enolate-forming species in the CS active site.[a]

System[b]	$\Delta\Delta E_{act}$	$\Delta\Delta E_{react}$
Full QM/MM	1.80 ± 0.33	2.05 ± 0.37
FaCoA(QM)	1.67 ± 0.57	2.51 ± 0.35
FaCoA(QM) + Asp375	0.92 ± 0.25	1.23 ± 0.25
FaCoA(QM) + Asp375 + OAA	0.97 ± 0.57	1.29 ± 0.96

[a] Average single-point SCS-MP2 QM(/MM) energy differences in kcal mol^{-1} from five different conformations (with standard deviations), between reactant and TS ($\Delta\Delta E_{act}$) and reactant and enolate ($\Delta\Delta E_{react}$). Conformations were extracted from the full QM/MM-optimized system. [b] Systems evaluated, respectively: Full QM/MM model, part of FaCoA treated QM, the same with Asp375 side chain, the same with Asp375 side chain and OAA.

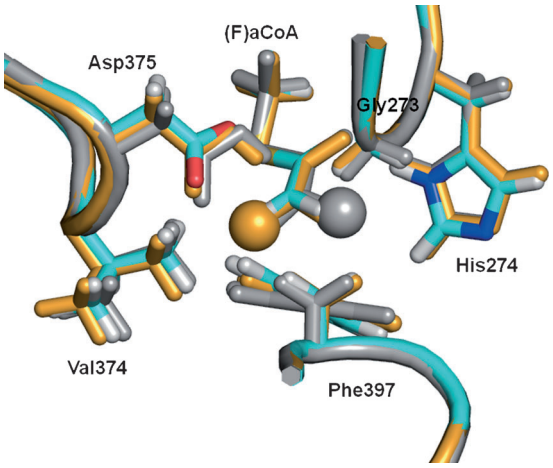

Figure 3. Structural comparison of typical enolate intermediates obtained from high-level QM/MM modeling. Enolate of acetyl-CoA from Ref. [8] (C cyan, O red, S yellow, H white) and *E*-enolate (gray) and *Z*-enolate (orange).

the subtlety of energetic and binding effects and the complexity of enzymes. Here, we have shown that an archetypal enzyme selectivity problem, the enantioselective conversion of fluoroacetyl-CoA to fluorocitrate by CS, crucial for the toxicity of fluoroacetate, can be successfully explained using high-level QM/MM modeling. The calculated energy differences in the enzyme-bound *E*- and *Z*-enolates quantitatively agree with experimental observations. The results indicate that the inherent energy difference between the enolates is the main cause of the observed selectivity. This study demonstrates how high-level QM/MM modeling can contribute to analyzing stereoselectivity in enzymes. These findings also further support the proposal that the reaction in CS involves an enolate intermediate,[7–9,12] in contrast to some previous studies and popular textbooks that suggest an enol instead.[10,14]

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