Stereospecific Control of the Citrate Synthase Mediated Synthesis of (2*R*,3*R*)-3-Fluorocitrate by the Relative Stabilities of the Intermediate Fluoroenolates

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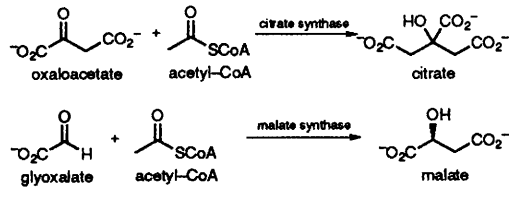
A model for the stereochemical course of enzymatically mediated condensations involving fluoroacetyl–CoA is provided by *ab initio* calculations of the relative stabilities of the *E*- and *Z*-isomers of fluorothioacyl enols and enolates, in which we suggest the enolate intermediate is substantially protonated for citrate synthase but not for malate synthase.

The toxicity of fluoroacetate is attributed to its *in vivo* activation to fluoroacetyl–CoA and its subsequent conversion to (2*R*,3*R*)-fluorocitrate† by the action of citrate synthase.[1,2] The accumulation of (2*R*,3*R*)-fluorocitrate inhibits the citric acid cycle enzyme aconitase,[3] and citrate transport more generally in cells,[4,5] and rapidly induces respiratory failure in mammals. It is an interesting quirk that citrate synthase mediates the formation of (2*R*,3*R*)-fluorocitrate, the only toxic stereoisomer of the four possible forms.[6] During the condensation with oxaloacetate (Scheme 1), citrate synthase makes a clear distinction between the two prochiral hydrogens of fluoroacetyl–CoA and abstracts with high selectivity ($\Delta G >$ 3 kcal mol^{-1}) only the 2-*pro*-*S* hydrogen atom. The condensation proceeds with inversion of configuration at this centre and attack is to the *Si*-face of the α-carbonyl of oxaloacetate.

Malate synthase is another example where fluoroacetyl–CoA can replace acetyl–CoA in an enzyme mediated condensation.[7,8] For malate synthase both (3*R*)- and (3*S*)-fluoromalates are generated from fluoroacetyl–CoA and glyoxal in a diastereoisomeric ratio of 4:3. In essence the enzyme displays a slight preference for abstraction of the 2-*pro*-*R* over the 2-*pro*-*S* hydrogen of fluoroacetyl–CoA. When (2*R*)-[2-^{2}H]-fluoroacetyl-CoA, carrying deuterium in the 2-*pro*-*R* position, was incubated with the enzyme then the diastereoisomeric bias changed to 3:7, now with a preference for 2-*pro*-*S* proton abstraction due to an isotope effect. Therefore for malate synthase, unlike citrate synthase, the enzyme is showing a limited ability to orientate the fluorine atom. As part of a more general programme[9] focused on evaluating the stereoelectronic influence of fluorine in enzymatic transformations we became interested in the origin of the diastereoselectivity displayed by citrate synthase with fluoroacetyl–CoA.

It is widely appreciated that fluorine exhibits a limited steric influence over hydrogen in enzyme reactions.[10] It has been suggested[11] that the selectivity of the citrate synthase reaction may be attributed to a F···H hydrogen bond anchoring the fluorine to the enzyme and hence favouring one of the orientations. Such an argument could be extended to malate synthase by invoking a weaker hydrogen bond at the active site of that enzyme. This is however a tenuous argument as fluorine forms only weak hydrogen bonds.[12,13] It may be however that at a geometrically ordered, and desolvated active site, the strength of a directed hydrogen bond or interaction with an alkali metal cation would be maximal and sufficient to contribute to the overall stability of a particular transition state conformation. This hypothesis therefore remains to be tested.



Scheme 1

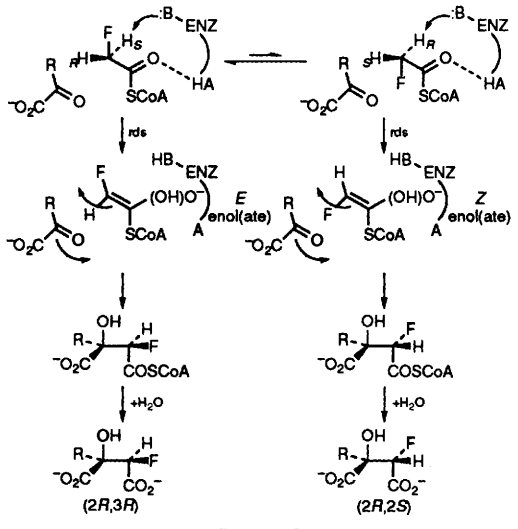
For the two enzymes under discussion, kinetic isotope data (citrate synthase[14] k_H/k_D = 1.94, malate synthase[15] k_H/k_D = 3.9) suggest that the rate-limiting step in both cases is proton abstraction from acetyl–SCoA. This is consistent with a much earlier deduction for citrate synthase, that the rate limiting event is the generation of an enolate intermediate.[16] Therefore when fluoroacetyl–CoA is used as a substrate in these enzymes (Scheme 2, R = $CH_2CO_2^-$ for citrate synthase, R = H for malate synthase), the prochiral hydrogen discrimination by the enzyme must be related to the relative energies of the *syn* or *anti* orientations of the C–F bond with respect to the oxygen atom of the forming neutral enol or enolate anion intermediate, assuming specific binding for the SCoA moiety. We have selected to focus on the energies of the different possible enol/enolate intermediates formed directly after the rate limiting step, with the assumption that these will be directly related to the relative energies of the transition states for proton abstraction.

The relative energies of the two isomers were studied using quantitative molecular orbital theory at the *ab initio* SCF-MO level, for which reliable calculations for molecules of this size can be made.‡ To enable the use of a large basis set, the SCoA

Table 1 Relative calculated energies of enols and enolates

	Enol[a]	Enolate anion[b]
E/MP2	−650.1865 (−650.1847)[c]	−649.5732
E/BLYP	−651.1781	−650.6561
Z/MP2	−650.1797	−649.5715
Z/BLYP	−651.1712	−650.6534

[a] Fluorothioacyl enol. Energies in Hartree at the 6-31 (3d) basis set level with correlation energy corrections at either the MP2 or BLYP level. [b] Fluorothioacyl enolate anion. Energies in Hartree at the 6-31 +(3d) basis set level. [c] OH group anti to C=C bond.



Scheme 2

component was modelled with SH. For the anionic enolates, we used a 6-31 + (3d) basis set, which includes a diffuse description for the anion. The neutral enol was studied using a 6-31(3d) basis. Correlation energy corrections were included at either the MP2 level or using exchange and correlation density functionals (B-LYP terms), and full geometry optimisation on all species at these levels was performed (Table 1). Zero-point vibrational corrections did not significantly influence the relative energies at the RHF/6-31(3d) level, and hence were ignored at the computationally more expensive MP2 or BLYP levels. The results show good agreement between the MP2 and B-LYP levels, and clearly indicate that the *E* enol is significantly more stable by 4.3 kcal mol^{-1} than the *Z* isomer, an energy difference that is reduced to 1.1 (MP2) or 1.9 (B-LYP) for the anionic enolate system. Two orientations (*syn* and *anti* to the C=C bond) of the OH bond in the neutral *E* enol were significantly lower than the *Z* form, suggesting the stabilisation is not due to any intramolecular O–H···F hydrogen bond[18] but is rather an example of the *cis* effect as exhibited by *e.g. cis* difluoroethene.[19]

This study has established that at a high level of theory, fluoroacetate thioesters are deprotonated preferentially to generate *E* enols or to a lesser extent *E* enolates. A straightforward but striking conclusion to emerge is that the degree of protonation of the enolate modulates the energy difference between the *E* and *Z* geometries. These results therefore suggest a model in which citrate synthase acts not only as a general base in removing the proton, but also as a general acid in protonating the enol[20] to achieve a selectivity of greater than 100 : 1 in favour of the observed stereoisomer.

Our hypothesis extends to malate synthase if it is assumed that this enzyme stabilises an intermediate with greater enolate than enol character. The resultant *E* and *Z* enolates are now much closer in energy and both potential diastereoisomers of fluoromalate will form. The observed stereochemical preference (4 : 3) is opposite to that predicted but other minor factors (*e.g.* steric, dipolar/electrostatic interactions, F···H-bonding *etc.*) may contribute and push the bias over in the other direction. An alternative explanation for this diastereoisomeric mixture is flexibility at the enzyme surface in orientating the C=O and SCoA groups. However such a lack of specificity in binding a coenzyme-A thioester is contra intuitive and at present we prefer to interpret our results as illustrated in Scheme 2, where O and S remain fixed but that the energy of the *E* and *Z* enolates is similar. We note that these models do not rely on a C–F···enzyme hydrogen bond.

Additionally, our model for citrate synthase helps to define a three-dimensional relationship between the general base in the enzyme, the binding site for the SCoA group, and the 2-*pro-S* hydrogen of the fluoroacetyl–SCoA. A further spatial descriptor, the possible general acid site is thus defined in this model. This general acid can be strategically placed to protonate the carbonyl of oxaloacetate with the proton contributing to a six-membered transition state for the C–C condensation, as illustrated in Fig. 1, the next step in the process. It is noteworthy in this respect that citrate synthase will catalyse exchange of the protons of acetyl–CoA when L-malate (but not D) replaces oxaloacetate and that no exchange occurs without L-malate.[22] Clearly L-malate could replace oxaloacetate in Fig. 1 and assist the strategic placement of the general acid,[23] through hydrogen bonding, for stabilisation of the developing enol intermediate.

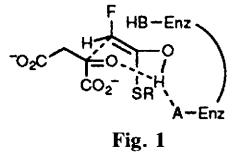


Fig. 1

Added in proof: Recent experimental results (D. P. Martin *et al.*, *J. Am. Chem. Soc.*, 1994, **116**, 4660) have also concluded an enol intermediate as the nucleophilic species in the citrate synthase reaction.

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Footnotes

† The numbering system is that which has developed historically and refers to citric acid as a parent, and is not here altered by the fluorine substituent. See ref. 8 for further discussion.

‡ Calculations were performed using the G2 release of Gaussian 92/LDF,[17] with full geometry optimisation using the Eigenvector following method. The final molecular coordinates in chemical MIME format[21] are available *via* the world-wide-web server (www) using the URL http://www.ch.ic.ac.uk/rzepa/CC/4_02941K.html. To connect to this service, use a www browser such as NCSA Mosaic or MacWeb/WinWeb.

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