Kinetic and Stereoelectronic Effects of a Fluorine Substituent on the Reaction Catalysed by an NADPH-Dependent Cyclohex-1-enylcarbonyl CoA Reductase

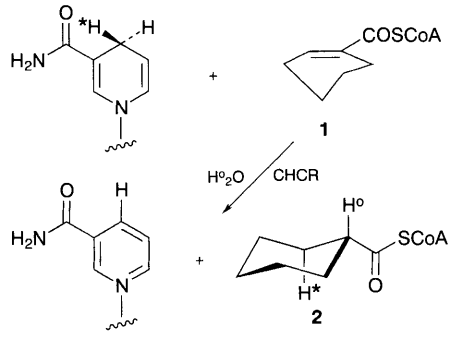
Colin F. Bridge,*[a] David O'Hagan,*[a] Kevin A. Reynolds[b] and Kimberlee K. Wallace[b]

[a] *Department of Chemistry, Science Laboratories, University of Durham, Durham, UK DH1 3LE*
[b] *Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, Md 21201, USA*

The introduction of a fluorine atom at C-3 of cyclohex-1-enecarbonyl CoA has a dramatic effect on the processing of the fluorinated over the natural analogue by NADPH dependant cyclohex-1-enecarbonyl CoA reductase from *Streptomyces collinus;* the fluorinated analogue is processed with a five fold increase in V_{max} and kinetic isotope studies suggest that hydride delivery is only partially rate limiting in the latter case; the enzyme also shows a small kinetic preference for axial over equatorial fluorine at C-3 of 3-fluorocyclohex-1-encarbonyl CoA **4**.

There have been extensive kinetic and mechanistic studies carried out on NAD(P)H-dependent alcohol dehydrogenases.[1] By contrast NAD(P)H-dependent enoyl thioester reductase have received limited study although such reactions play a key role in processes such as fatty acid and polyketide biosynthesis.[2]

NADPH dependant cyclohex-1-enylcarbonyl CoA reductase (CHCR) catalyses the reduction of cyclohex-1-enylcarbonyl CoA **1** to cyclohexylcarbonyl CoA **2** (Fig. 1) and is thought to be responsible for formation of a cyclohexyl moiety of the antifungal agent ansatrienin produced by *Streptomyces collinus*.[3] This enzyme has recently been cloned, overexpressed in *Escherichia coli.* and purified to homogeneity.[4] The stereochemical course of the reduction proceeds with transfer of a hydrogen from the *pro*-4*S* position of NADPH to the *Si* face of C-2 and *anti* incorporation of solvent hydrogen at C-1.[5] As part of our ongoing studies on the effects of selective fluorination on enzyme reactions[6–8] we have investigated 3-fluorocyclohex-1-enecarbonyl-CoA **4** as a substrate for CHCR. The cyclohexane ring system presents an ideal situation in which to explore stereoelectronic effects of fluorine substitution. Here we report that incorporation of a fluorine substituent at C-3 of the substrate dramatically enhances the catalytic rate of the reaction, and has an effect on the rate limiting step of the process. Furthermore CHCR exhibits a small kinetic differentiation between the two diastereoisomers of 3-fluorocyclohex-1-enylcarbonyl CoA **4**.



Scheme 1 Stereochemical course for the conversion of **1** into **2** catalysed by cyclohex-1-enecarbonyl-CoA reductase

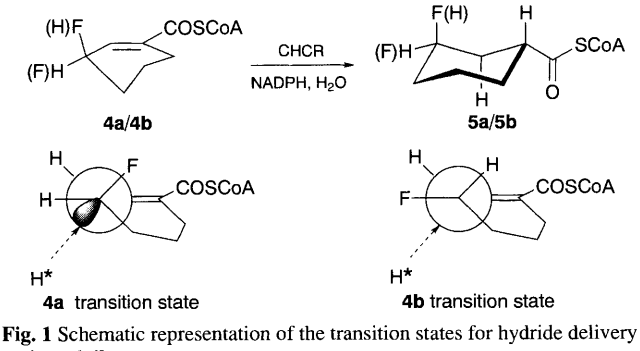


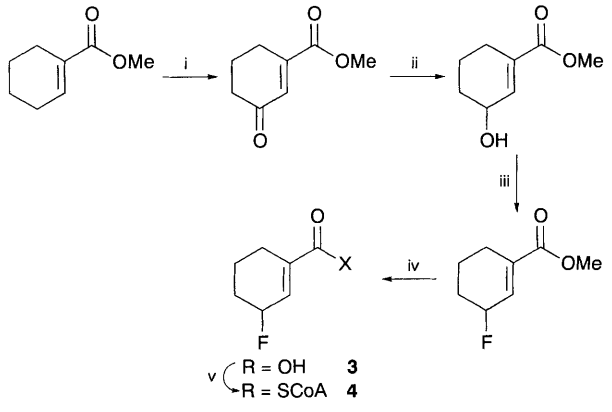
Fig. 1 Schematic representation of the transition states for hydride delivery to **4a** and **4b**

Racemic 3-fluorocyclohex-1-enecarboxylic acid **3**† was synthesized from methyl cyclohex-1-enecarboxylate as shown in Scheme 2. The coenzyme-A ester **4** was prepared[12] and was used directly in enzyme assays. The K_m and V_{max} values were obtained for the natural substrate cyclohex-1-enylcarbonyl CoA **1** with purified CHCR using NADPH and [4*S*-^{2}H]NADPH (Table 1).[13]

The decrease in the V_{max} of the reaction using [4*S*-^{2}H]NADPH is indicative of a primary isotope effect (k_H/k_D = 3.0) and shows that this step is significantly rate limiting. Similar primary isotope effects (k_H/k_D = 3–5) have been observed in NAD(P)$^+$ dependent alcohol dehydrogenases where hydride (deuteride) transfer between the substrate and nucleotide cofactor is the rate limiting step of the reaction.[14,15] The decreased K_m for [4*S*-^{2}H]NADPH compared to NADPH is unlikely to be due to an isotope effect on binding. Rather, the observation of an isotope effect on K_m under conditions where there are substantial isotope effects on V_{max} likely indicates a Michaelis constant that is a steady state rather than a dissociation constant.

The V_{max} for the reaction catalysed by CHCR with 3-fluorocyclohex-1-enylcarbonyl CoA (**4a** and **4b**) was almost five-fold greater than that with the natural substrate **1**. A rate enhancement of this magnitude by addition of a fluorine substituent in an enzyme catalysed reaction is unprecendented[16] but presumably arises due to the electronegativity of the fluorine atom at C-3 lowering the energy barrier for hydride delivery to C-2.‡

The K_m value (Table 1) for **4** was higher than **1** although it still emerges as an excellent substrate. The K_m values determined for NADPH was also higher for **4** than **1** and those determined for [4*S*-^{2}H]NADPH were almost seven fold greater with **4** than **1**. These increases are accompanied by an increased reaction rate suggesting that the Michaelis constant for the nucleotide cofactor is a steady state rather than a dissociation constant. Significantly the V_{max} values obtained for **4** using



Scheme 2 *Reagents and conditions*: i, CrO_3, Ac_2O, AcOH, benzene,[9] 56%; ii, $NaBH_4$, $CeCl_3.7H_2O$, MeOH,[10] 80%; iii, diethylaminosulfur trifluoride,[11] CH_2Cl_2, 69%; iv, KOH, MeOH, 77%; v, CoASH, Et_3N, EtOCOCl (quantitative)

Table 1 Kinetic analyses of the reaction catalysed by cyclohex-1-enylcarbonyl CoA reductase using NADPH and NADP^{2}H. Substrate concentrations ranged from 3 to 100 μmol dm^{-3} and data were analysed by non-linear regression analysis.

Substrate	Cofactor	Substrate K_m[a]/μ (dm^3 mol^{-1})	Cofactor K_m[b]/μ (dm^3 mol^{-1})	V_{max}[c]/U mg^{-1} protein
Cyclohex-1-enylcarbonyl CoA **1**	NADPH	25 ± 1	9.5 ± 1	7.5 ± 0.5
	NADP^{2}H	26 ± 4[d,f]	3.6 ± 0.9	2.5 ± 1.0
3-Fluorocyclohex-1-enylcarbonyl CoA **4**	NADPH	44 ± 4	16 ± 3[e,f]	36 ± 5
	NADP^{2}H	14 ± 4	28 ± 4	27 ± 1

[a] Reported substrate K_m values are averages obtained from analyses conducted at 50 and 100 mmol dm^{-3} cofactor concentrations. [b] Reported cofactor K_m values are averages obtained from analyses conducted at 100 and 200 mmol dm^{-3} substrate concentrations. [c] V_{max} values are average values obtained from all K_m determinations. One unit (U) is defined as the oxidation of 1 μmol of NADPH per min. [d] Determined in duplicate only at a cofactor concentration of 100 mmol dm^{-3}. [e] Determined in duplicate only at a substrate concentration of 100 μmol dm^{-3}. [f] Estimated errors.

NADPH and [4*S*-^{2}H]NADPH were much closer (k_H/k_D *ca.* 1.3) than those for **1** (k_H/k_D *ca.* 3) indicating that the hydride (deuteride) transfer is at best only partially rate limiting. Presumably the fluorine substituent has lowered the energy barrier for hydride delivery sufficiently such that another step in the catalytic cycle is contributing significantly to the overall rate. The V_{max} for the reduction of **4** by CHCR in D_2O rather than H_2O was unchanged indicating that the proton transfer is not rate limiting in the fluorinated analogue. Accordingly, the rate-limiting step for the reduction of **4** by CHCR is either substrate/cofactor binding or product/cofactor release.[17] As hydride transfer is clearly rate limiting for reduction of **1** but not **4**, the difference in V_{max} for the reduction of these substrates with [(4*S*)-^{2}H]NADPH (approximately 15-fold) is even more dramatic than with NADPH.

Interestingly, CHCR exhibited a kinetic differentiation between the two diastereoisomers of **4** (**4a** and **4b**). Assuming that the enzymatic reduction of **4** proceeds with the same stereospecificity as **1**, then the preference for the COCoA moiety to lie equatorially, dictates that one epimer (**4a**) generates product with fluorine axial (*i.e.* **5a**) and the other epimer (**4b**) generates product with fluorine equatorial (*i.e.* **5b**). ^{19}F{^{1}H}-NMR analysis of the CHCR reduction of **4** with time revealed a steady disappearance of the substrate signal at δ −171 and a concomitant increase in two signals at −182.5 and −168.5 which could be assigned[18] to the formation of (3*R*)-fluoro-(1*R*)-cyclohexylcarbonyl CoA **5a** (axial fluorine) and (3*S*)-fluoro-(1*R*)-cyclohexylcarbonyl CoA **4b** (equatorial fluorine), respectively. After 20–30% conversion the ratio of integrals of the ^{19}F{^{1}H}-NMR signals associated with **5a** and **5b** was approximately 1.3 : 1 (3 runs) and then this ratio steadily decreased to 1 : 1 as the reaction was allowed to run to completion. Clearly both **4a** and **4b** react faster than **1** with CHCR due to an inductive effect, but there is also a small kinetic preference for the production of **5a** over **5b** in the early stages of the reaction. This may be a binding effect, but would require that the K_m values of **4a** and **4b** are significantly different with the experimental K_m value emerging as an average of the two. Alternatively theoretical analysis of nucleophilic attack at sp^2 hybridized carbons with fluorine at the α-carbon suggests that the preferred transition state geometry has fluorine anti-periplanar to the incoming nucleo-phile due to an n-σ* stabilizing interaction of the Anh–Eisenstein type.[6,19,20] The hydride addition by CHCR to the *Si*-face of C-2 of **1** suggests that the transition state for hydride delivery to **4a** (fluorine axial) rather than **4b** (fluorine equatorial) will stabilize the incoming hydride more effectively due to donation of negative density into the σ* orbital associated with the fluorine atom (lobe transition state **4a** in Fig. 1). This rational is consistent with the experimental observation.

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Footnotes

† *Selected physical and spectroscopic* data for **3**. Mp 62–64 °C; δ_H ($CDCl_3$, 400 MHz) 11.05 (1 H, bs, CO_2H), 7.04 (1 H, dm, CH), 5.515 (1 H, dm, J_{HF} = 47 Hz) and 1.55–2.5 (6 H, m, CH_2); δ_C($CDCl_3$) 172.3 (CO), 137.0 (d, $^2J_{CF}$ = 19.6 Hz, =CH), 134.5 (d, $^3J_{CF}$ = 9.3 Hz, =C), 85.7 (d, J_{CF} = 164.8 Hz, CHF), 28.2 (d, $^2J_{CF}$ = 19.3 Hz, CH_2), 23.8 (d, $^4J_{CF}$ = 2.7 Hz, CH_2) and 18.1 (d, $^3J_{CF}$ = 2.6 Hz, CH_2); δ_F ($CDCl_3$, 376 MHz) and −173.3 (d, J_{FH} = 45 Hz); *m/z* (EI$^+$) 144.1 (M^+, 29.5%).

‡ An increase in rate of hydride transfer from NADH to an aldehyde by electron withdrawing groups has previously been observed with the NAD$^+$ dependent yeast alcohol dehydrogenase.[14] However, all of the substituted benzaldehyde derivatives studied were converted much more slowly than the natural acetaldehyde substrate.

References

1 J. P. Klinman, *C.R.C. Crit. Rev. Biochem.*, 1981, **10**, W. W. Cleland, *Methods Enzymol.*, 1980, **64**, 104; J. P. Klinman, *Biochemistry*, 1976, **15**, 2018.
2 U. G. Wagner, H. Bergler, S. Fuchsbichler, F. Turnowsky, G. Hogenauer and C. Kratky, *J. Mol. Biol.*, 1994, **243**, 126; J. B. Rafferty, J. W. Simon, A. R. Stuitje, A. R. Slabas, T. Fawcett and D. W. Rice, *J. Mol. Biol.*, 1994, **237**, 240.
3 K. A. Reynolds, P. Wang, K. M. Fox, M. K. Speedie, Y. Lam and H. G. Floss, *J. Bacteriol.*, 1992, **14**, 3850.
4 K. Reynolds and K. Wallace, unpublished results.
5 K. A. Reynolds, K. M. Fox, Z. Yuan and Y. Lam, *J. Am. Chem. Soc.*, 1991, **113**, 4339.
6 D. O'Hagan and H. S. Rzepa, *J. Chem. Soc., Perkin Trans. 2*, 1994, 3.
7 D. O'Hagan and H. S. Rzepa, *J. Chem. Soc., Chem. Commun.*, 1994, 2029.
8 L. Dasaradhi and D. O'Hagan, *Bioorg. Med. Chem. Lett.*, 1993, **3**, 1655.
9 G. L. Lange and J. A. Otulakowski, *J. Org. Chem.*, 1982, **47**, 5093.
10 A. L. Gemal and J-L. Luche, *J. Am. Chem. Soc.*, 1981, **103**, 5454.
11 W. J. Middleton, *J. Org. Chem.*, 1975, **40**, 574.
12 M. Lai, D. Li, E. Oh and H. Liu, *J. Am. Chem. Soc.*, 1993, **115**, 1620.
13 G. Ottolina, S. Riva, G. Carrea, B. Daneli and B. F. Buckman, *Biochim. Biophys. Acta*, 1989, **998**, 173.
14 J. P. Klinman, *J. Biol. Chem.*, 1972, **247**, 7977.
15 A. Fersht, *Enzyme Structure and Mechanism*, W. H. Freeman and Co., New York, 2nd edn 1985; Ch. 2 and 15.
16 K. E. Stremler and C. D. Poulter, *J. Am. Chem. Soc.*, 1987, **109**, 5542.
17 The rate limiting step in the NAD$^+$-dependent horse liver alcohol dehydrogenase catalysed reaction, which has a k_H/k_D = 1 for the hydride step, is the release of the NAD$^+$ cofactor. K. Dalziel, *J. Biol. Chem.*, 1963, **238**, 2850; C. C. Wratten and W. W. Cleland, *Biochemistry*, 1963, **2**, 935; 1965. **4**, 2442.
18 The chemical shifts in the ^{19}F{^{1}H} spectra for axial and equatorial fluorines were consistent with literature values, see: *e.g.* J. Cantacuzene and R. Jantzen, *Tetrahedron Lett.*, 1970, **37**, 3281. These assignments were reinforced by the magnitude of the proton fluorine coupling constants in the ^{19}F NMR spectrum; Equatorial (δ −168.5, broad doublet, J = 47 Hz) and axial (δ −182.5, doublet of broad triplets, J = 47 and 30 Hz).
19 S. S. Wong and M. N. Padden-Row, *J. Chem. Soc., Chem. Commun.*, 1990, 456.
20 N. T. Anh and O. Eisenstein, *Nouv. J. Chim.*, 1977, **1**, 61.