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The perils of rational design – unexpected irreversible elimination of fluoride from 3-fluoro-2-methylacyl-CoA esters catalysed by α-methylacyl-CoA racemase (AMACR; P504S)†

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α-Methylacyl-CoA racemase (AMACR; P504S) catalyses 'racemization' of 2-methylacyl-CoAs, the activation of *R*-ibuprofen and is a promising cancer drug target. Human recombinant AMACR 1A catalyses elimination of 3-fluoro-2-methyldecanoyl-CoAs to give *E*-2-methyldec-2-enoyl-CoA and fluoride anion, a previously unknown reaction. 'Racemization' of 2-methyldec-3-enoyl-CoAs was also catalysed, without double bond migration.

α-Methylacyl-CoA racemase (AMACR,‡ P504S; E.C. 5.1.99.4) catalyses the key 'racemization' step in the degradation of branched-chain fatty acids and is also important in the pharmacological activation of *R*-ibuprofen and related drugs.[1–3] The enzyme catalyses the conversion of either epimer of a 2-methylacyl-CoA ester to a *ca.* 1 : 1 mixture of **2*R***- and **2*S***-epimers.[4] AMACR has also been proposed to be involved in the uni-directional chiral inversion of mandelic acid in mammals[5] but this was recently shown to proceed by a distinct pathway.[6]

AMACR protein levels and enzyme activity are increased in prostate cancers[7,8] a subset of colon cancers[9] and various other cancers[1] and it is widely recognised as a promising new drug target.[1,2,10,11] However, relatively few chemical inhibitors of AMACR have been reported,[12–15] largely due to the lack of a convenient, high-throughput assay.

No X-ray crystal structure for a mammalian AMACR has been reported but the enzyme is proposed to catalyse its reaction by removal of the α-proton by Asp-152 or the His-122/Glu-237 pair[1,2,4,16,17] to form an enolate intermediate.[18] Non-stereoselective reprotonation of the enolate gives a ~1 : 1 mixture of 2-methylacyl-CoAs with the original and epimeric configuration at the α-carbon.[2,4] Enolates are common intermediates in a number of enzymatic reactions, including condensations,[19] double-bond migrations[20] and elimination reactions,[21] and AMACR could potentially perform these reactions with the appropriate substrates. This *communication* reports that human recombinant AMACR 1A[4] is able to catalyse an elimination reaction with 3-fluoro-2-methylacyl-CoA substrates to give the corresponding unsaturated 2-methylacyl-CoA ester and a fluoride anion.[22] It is also able to catalyse 'racemization' of unsaturated 2-methylacyl-CoA esters but does not catalyse migration of the double bond.

The known substrate 2-methyldecanoyl-CoA **1*S*** allows the course of the enzyme reaction to be followed using proton/deuterium exchange in ^2H_2O. However this assay only yields information on exchange of the α-proton; to obtain information on the stereochemistry a time consuming and scale-limited derivatization of products is needed. It was hoped that using fluorinated analogues of 2-methylacyl-CoA esters would overcome this problem. Specifically *syn*- and *anti*-3-fluoro-2-methyldecanoyl-CoA, **2*S*** and **2*R***, were chosen as it was anticipated that chiral inversion and α-proton exchange of this epimeric pair of substrates could be directly and simultaneously observed by changes in the ^1H and ^{19}F NMR spectra. *S*- and *R*-*E*-2-Methyldec-3-enoyl-CoAs **3*S*** and **3*R*** were chosen for testing in order to determine whether AMACR could catalyse double bond migration into conjugation with the carbonyl group, whilst *E*-2-methyldec-2-enoyl-CoA **4** was selected as the proposed product of this reaction (Fig. 1).

The required substrates were synthesised by extension of reported methods.[3,4,12] *anti*-3-Fluoro-2-methyldecanoic acid **5** was synthesised by the method of Carnell *et al.*[12] using octanal (ESI,† Scheme S2). *syn*-3-Fluoro-2-methyldecanoic acid **6** was synthesised by aldol reaction of *N*-propanoyl-Evans' auxiliary with 2-octenal, followed by hydrogenation, conversion to the methyl ester, treatment with DAST[12] and deprotection (ESI,† Scheme S3). *S*- and *R*-2-Methyldec-3-enoic acids **7** were synthesised by reaction of the Grignard reagent derived from *E*-1-crotyl chloride **8** with CO_2, followed by chiral resolution of the resulting acids as the *N*-acylated *R*-Evans' auxiliary derivatives. Metathesis with 1-octene and deprotection gave the required unsaturated acids (ESI,† Scheme S4). 2-Methyldec-2-enoic acid **9** was synthesised by a Wittig reaction between octanal and the ylide derived from ethyl 2-bromopropanoate and Ph_3P, followed by deprotection

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† Electronic supplementary information (ESI) available: Assignment of stereochemical configurations and description of reactions; synthetic methods; enzyme kinetic plots. See DOI: 10.1039/c4cc06127f

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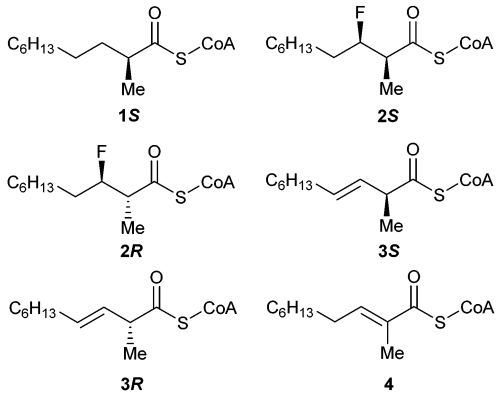

Fig. 1 Structures of the acyl-CoA esters incubated with human recombinant AMACR 1A.

(ESI,† Scheme S5). These acids were converted into their corresponding acyl-CoA esters by activation with *N,N′*-carbonyldiimidazole followed by reaction with CoA-SH.[3]

Initially, acyl-CoA esters were incubated with human recombinant AMACR 1A[4] in the presence of ^2H_2O. Incubation of **2*R*** and **2*S*** with active AMACR was expected to result in formation of a mixture of epimers at carbon-2 with exchange of the α-proton for deuterium resulting in formation of a broad single peak (2-bond coupling to the ^2H is not normally observed). Unexpectedly, the peak at *ca.* 1.1 ppm diminished with time (Fig. 2) and a new singlet at *ca.* 1.75 ppm simultaneously arose and increased in intensity. In the ^{19}F spectrum the signal for **2*R*** and **2*S*** slowly disappeared and a new signal appeared at δ −122 ppm, which is characteristic of inorganic fluoride. Taken together these observations suggested that an elimination reaction had occurred. Product levels increased over time when **2*S*** or **2*R*** were incubated with active AMACR (ESI,† Fig. S1). For **2*R***, this reaction was not observed in negative controls containing heat-inactivated enzyme.

Comparison of the ^1H NMR spectrum of the unsaturated acyl-CoA product from the enzymatic elimination reaction showed that it was identical to **4**. Compound **4** had been synthesised from the corresponding *E*-acid, as assigned by the alkene proton signal at δ 6.92 ppm (The *Z*-acid alkene proton appears at 6.09 ppm).[23]

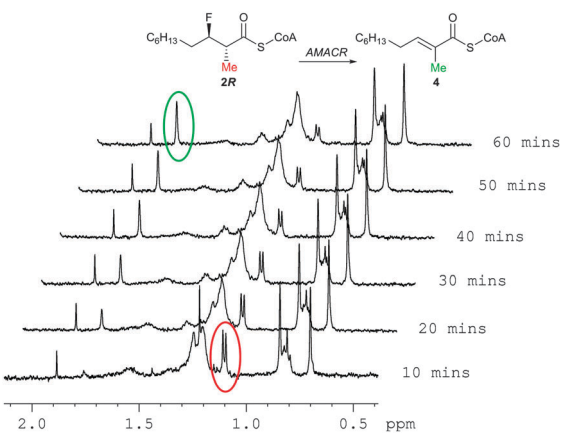

Fig. 2 ^1H NMR spectrum of **2*R*** when incubated with AMACR over time.

This shows that the geometry of the double bond of the precursor acid can be assigned and confirms that the product of the enzymatic reaction is the *E*-isomer.

Both **2*S*** and **2*R*** gave the same *E*-acid product **4**, consistent with an E1cb mechanism[21] in which an enolate intermediate[18] is used to expel the fluoride. The resulting *E*-double bond suggests that the reaction occurs with the substrate in an *anti*-conformation with respect to the α-proton and fluoride. This contrasts with enoyl-CoA hydratase,[21,24] which catalyses its E1cb reaction with *syn*-elimination because the two catalytic glutamate residues are on the same face of the substrate.[24] *anti*-elimination by AMACR probably results from the combination of a number of factors:[21] firstly, the substrate side-chain is bound by a hydrophobic surface,[17] allowing adoption of the more favourable *anti*-conformation; secondly, fluorine is a relatively small substituent;[25] and third, fluoride is likely to be highly solvated in aqueous solution. Work in similar chemical systems suggests that fluoride is eliminated by an E1cb-like E2 mechanism[26,27] with *anti*-stereochemistry. It is also notable that other enzymes with enolate or enediol intermediates also eliminate HF from substrate analogues, including butyryl-CoA dehydrogenase,[28] uronate isomerase[29] and glyoxalase I.[30] Some of these enzymes[28,29] also possess active-site aspartate or glutamate residues acting as bases.

Kinetic analysis of the AMACR-catalysed elimination reaction showed that Michaelis–Menten behaviour was observed. The following kinetic parameters were determined for **2*R*** by the Direct Linear Plot:[31,32] K_m = 21 μM; V_{max} = 96.5 nmol min^{-1} mg^{-1} protein; k_{cat} = 0.0758 s^{-1}; k_{cat}/K_m = 3612 M^{-1} s^{-1}. This compares to K_m = 277 μM; V_{max} = 39.3 nmol min^{-1} mg^{-1} protein; k_{cat} = 0.0310 s^{-1}; k_{cat}/K_m = 112 M^{-1} s^{-1} for *S*-2-methyldecanoyl-CoA **1*S***,[3] implying that the elimination reaction is ∼32× more efficient than the racemization reaction (as judged by k_{cat}/K_m). This is probably due to the electron-withdrawing effect of the fluorine atom increasing the α-proton acidity.[12] For **2*S***, significant background conversion was observed in negative controls, probably due to the *anti*-arrangement of the α-proton and fluorine atom in a favourable staggered conformation. The following approximate values for kinetic parameters were obtained for **2*S***: K_m = 40 μM; V_{max} = 50.6 nmol min^{-1} mg^{-1} protein; k_{cat} = 0.0397 s^{-1}; k_{cat}/K_m = 993 M^{-1} s^{-1}. **2*R*** needs to adopt a *gauche* conformation for *anti*-elimination, and hence the background non-enzymatic reaction is less favoured.

Incubation of **4** with active AMACR in the presence of fluoride did not show any conversion to **2**, showing that the elimination reaction is irreversible. Control experiments with ±-fenoprofenoyl-CoA showed that AMACR was equally active in the presence and absence of fluoride anions, showing the enzyme was not inactivated. The irreversibility of the elimination of **2** probably results from the high levels of hydration of the fluoride anion. Fluoride is also a hard nucleophile making it less likely to react with the soft conjugate electrophile.

Unsaturated 2-methylacyl-CoA esters were also investigated as substrates for AMACR. Incubation of **3*S*** and **3*R*** with active AMACR in the presence of ^2H_2O resulted in α-proton exchange, as judged by conversions of the doublet at δ *ca.* 1.1 (methyl) into a broad single peak and of the doublet of doublets at 5.29 ppm into a doublet in the ^1H NMR spectrum (ESI,† Fig. S2). Exchange of

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the α-proton is required for the chiral inversion of substrates by AMACR,[3,4] and therefore it is highly likely that chiral inversion has also taken place. Substrate incubated with heat-inactivated enzyme under the same conditions showed no changes, showing that AMACR catalyses 'racemization' of unsaturated substrates. Native human and rat enzymes have been previously reported[33,34] not to bind similar unsaturated acyl-CoA esters (based on a competition assay), suggesting they bind relatively weakly compared to saturated substrates.

Rearrangement of the double bond of **3*S*** or **3*R*** was not catalysed by AMACR, with no formation of **4** as shown by the absence of the characteristic methyl group singlet at *ca.* δ 1.8 in the ^{1}H NMR spectrum. These results imply that either no proton donor is in close proximity to the distal end of the double bond to facilitate migration or that reprotonation of the enolate intermediate[18] to give 'racemization' is much more efficient.

Incubation of the 2-unsaturated acyl-CoA ester **4** with active AMACR showed, by ^{1}H NMR analysis, that it was not converted to **3** or any other product. It is not clear whether this is due to **4** failing to bind to AMACR or if it binds but does not undergo a reaction. It is known that 2-methylene acyl-CoA esters, which also possess a sp^2-hybridised carbon-2, behave as reversible tight-binding dead-end inhibitors of human AMACR 1A *in vivo*,[2,13] suggesting that **4** may also be bound.

The results in this *Communication* demonstrate that human AMACR 1A is able to catalyse irreversible elimination of substrates, probably by an E1cb or E1cb-like E2 mechanism. The reaction is of potential utility for measuring the AMACR activity, since quantification of both the enoyl-CoA and fluoride products is possible. It is also notable that several AMACR inhibitors with similar structures to **2*R*** and **2*S*** have been reported.[12] Given that the only difference between these compounds and **2*R*** is the length of the side-chain, it is quite possible that these compounds also undergo an elimination reaction. Fluorine atoms are often used in drug molecules (with >20% of all drugs containing at least one fluorine atom[35]), but it is important to consider that they may be reactive under certain circumstances. These results also extend the range of substrates for the AMACR racemization reaction to include 2-methyl-3-enoyl-CoA esters.

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Notes and references

‡ Abbreviations used: AMACR, α-methylacyl-CoA racemase; CoA, coenzyme A; DAST, diethylaminosulfur trifluoride; ppm, parts per million.

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