Unexpected stereoselective exchange of straight-chain fatty acyl-CoA α-protons by human α-methylacyl-CoA racemase 1A (P504S)†

Fouzia A. Sattar, Daniel J. Darley, Francesco Politano, Timothy J. Woodman, Michael D. Threadgill and Matthew D. Lloyd*

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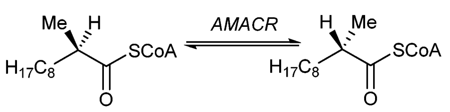
α-Methylacyl-CoA racemase (AMACR;‡ P504S) catalysed exchange of straight-chain fatty acyl-CoA α-protons. One α-proton was removed in each catalytic cycle, with the pro-*S* proton preferred. This reaction was most efficient for straight-chain substrates with longer side-chains. 2-Methyldecanoyl-CoA underwent α-proton exchange 3× more efficiently (as judged by K_{cat}/K_m) than decanoyl-CoA.

Branched-chain fatty acids, *e.g.* phytanic acid (3*R*/*S*,7*R*,11*R*,15-tetramethylhexadecanoic acid), are important components of the human diet and are also used as drugs *e.g.* ibuprofen.[1,2] Phytanic acid is derived from the phytol side chain of chlorophyll A and is abundant in red meat and dairy products.[3] High amounts of phytanic acid in the diet are a risk factor for prostate cancer,[4,5] the most common male-specific cancer. The presence of the 3-methyl group in phytanic acid prevents β-oxidation, and it is processed as its CoA ester by peroxisomal α-oxidation to give pristanic acid (2*R*/*S*,6*R*,10*R*,14-tetramethylpentadecanoic acid). Pristanic acid is metabolised as its CoA ester by β-oxidation in peroxisomes and subsequently in mitochondria for chain-shortened derivatives.[2,3,6] The β-oxidation pathway only oxidises α-methyl fatty acyl-CoA esters with 2*S* configuration,[2,7] but 2*R*-methylacyl-CoA esters are produced during the degradation of phytanic acid and other endogenous fatty acids.

Chiral inversion of these *R*-2-methylacyl-CoA esters is catalysed by the enzyme α-methylacyl-CoA racemase (AMACR) (Scheme 1),[1,2] and proceeds by removal of an α-proton to give an enol/enolate intermediate followed by non-stereoselective reprotonation.[1] The enzyme has two bases within the active site, the His-122/Glu-237 pair and Asp-156,



Scheme 1 Reaction catalysed by α-methylacyl-CoA racemase (AMACR), using *R*- and *S*-2-methyldecanoyl-CoA 1*R*, 1*S* as example substrates.

Medicinal Chemistry, Department of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, UK. E-mail: M.D.Lloyd@bath.ac.uk; Fax: +44 (0)1225-386114; Tel: +44 (0)1225-386786

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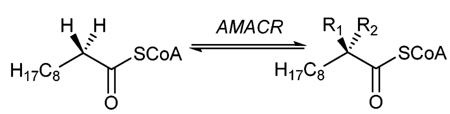
based on the structure of the *Mycobacterium tuberculosis* homologue, MCR.[8] The enzyme is an atypical two-base racemase, in that it incorporates a high level of deuterium into product in both directions.[1] This type of behaviour is more typical of one-base racemases. AMACR protein levels are increased in prostate cancer and it has attracted recent attention as a marker (P504S)[9,10] and potential drug target.[11–13]

Straight-chain fatty acyl-CoA esters are abundant in both peroxisomes and mitochondria, the compartments in which AMACR is localised. The strong resemblance to the natural substrates suggests that they might be potential substrates of AMACR. This communication reports that exchange of the α-protons of straight-chain fatty acyl-CoA esters is indeed catalysed by human AMACR 1A (Scheme 2). Specific questions addressed in this communication are: (1) Is one or are both α-protons exchanged during each catalytic event? (2) If only one α-proton is exchanged, is the reaction stereoselective? (3) Is the presence of substrate straight-chain fatty acyl-CoA esters likely to interfere with or modulate "conventional" racemase activity of AMACR *in vivo*?

Initially, decanoyl-CoA **2** was incubated with recombinant human AMACR 1A in buffer containing ^2H_2O. Decanoyl-CoA **2** was chosen as this is the straight-chain analogue of the previously reported *S*- and *R*-2-methyldecanoyl-CoA substrates (**1*S*** and **1*R***).[1] Incubations of **2** with active enzyme resulted in a reduction in the peak intensity of the triplet for the α-protons at δ 2.36–2.46 in the ^1H NMR spectrum, along with changes in the structure of the β-proton quintet at δ 1.40. Levels of conversion of *ca.* 35–40% were observed after incubation for 16 h. Incubation of 2,2′-[$^2H_2$]-decanoyl-CoA **3** (R^1, R^2 = ^2H) with active AMACR in ^1H_2O buffer resulted in the appearance of a triplet in the ^1H NMR spectrum for the α-protons and changes in the β-protons signal from a triplet into a quintet. High levels of conversion were observed, indicating that the α-^1H ↔ α-^2H exchange reactions are efficiently catalysed by AMACR in both directions. These



Scheme 2 Incorporation of deuterium during reaction of straight-chain fatty acyl-CoA esters (decanoyl-CoA **2**) with AMACR (R^1, R^2 = ^1H or ^2H).

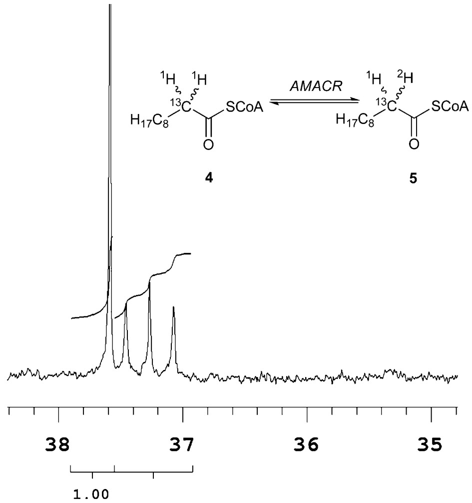


Fig. 1 ^{13}C NMR spectrum of 2-[^{13}C]-decanoyl-CoA **4** incubated with AMACR for 1 h at 30 °C, showing conversion of the substrate singlet at δ 37.65 to a triplet at δ 37.25, indicating replacement of one ^{1}H with ^{2}H.

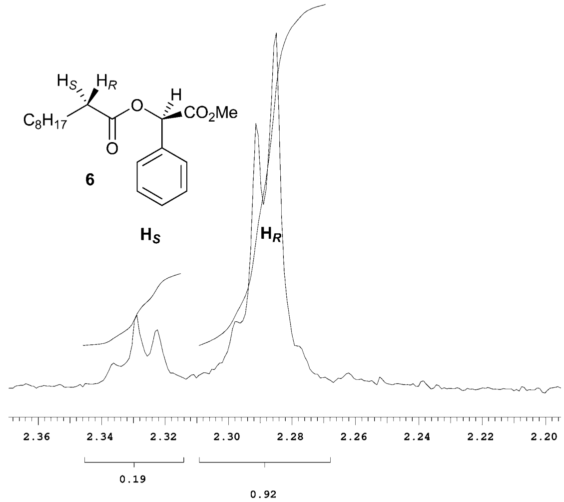


Fig. 2 ^{1}H NMR spectrum of monodeutero methyl *O*-decanoyl *R*-mandelates **6**.

changes were not observed in negative controls lacking active enzyme.

To determine whether one or both α-protons could be exchanged by AMACR in each catalytic cycle, incubations were carried out with 2-[^{13}C]-decanoyl-CoA **4** in $^{2}H_2O$ buffer. This resulted in exchange of the α-^{1}H for deuterium, as judged by ^{13}C NMR spectroscopy. At t = 0, the spectrum of the incubation mixture showed only a strong singlet at δ 37.65 arising from the 2-^{13}C carrying only 2 × ^{1}H. The ^{13}C NMR spectrum at early reaction time points also contained a triplet at δ 37.25, with peak-height ratio 1 : 1 : 1, indicating that only one deuterium was directly attached to the 2-^{13}C (Fig. 1). Only at very high levels of conversion was a small quintet (corresponding to 2-$^{13}C^{2}H_2$) seen in this region of the spectrum. These observations are only consistent with exchange of one α-proton at each "visit" of the substrate to the active site of the enzyme; the slow formation of 2-$^{13}C^{2}H_2$-decanoyl-CoA occurs from a second "visit" to the enzyme active site by 2-$^{13}C^{2}H_1$-decanoyl-CoA **5**. This result was initially surprising since the rate of exchange of protons was expected to be much faster than that of release of product, and hence exchange of both α-protons was expected. However, the proposed catalytic mechanism of AMACR[1,8] is consistent with substitution of a single proton. In this mechanism, one of the α-protons is removed by either Asp-152 or Glu-237/His-122, depending on whether the pro-*R* or pro-*S* proton is removed. The deprotonated intermediate can then react either with the proton on the initial base (to give back an unlabeled acyl-CoA) or with a deuterium on the other base. Extraction of the second α-proton can only take place if proton transfer occurs between the two catalytic bases or between the bases and bulk solvent before release of product.

The stereochemical course of this single substitution with deuterium was then investigated. Decanoyl-CoA **2** was incubated with active enzyme until *ca.* 60% exchange of the α-protons with deuterium had occurred. Following quenching of the reaction, the CoA ester mixture was hydrolysed and the resulting acids were derivatised with *R*-2-mandelate methyl ester. In unlabelled methyl *O*-decanoyl-*S*-mandelate, the 2-protons of the decanoyl unit are inequivalent and resonate at different frequencies in the ^{1}H NMR spectrum, with the pro-*S*-proton signal centered at δ 2.29 and the pro-*R*-proton at δ 2.33. These assignments were made by analogy with the findings of Parker[14] who showed that the pro-*R* 2-H of the aliphatic acyl group of a range of methyl *O*-(fatty-acyl)-*S*-mandelates always resonated at higher frequency than did the corresponding pro-*S* 2-H. Assignments of the more upfield and more downfield signals are reversed for chiral derivatization with methyl *R*-mandelate. Schwab and Lin[15] also used this method to establish the absolute configuration of synthetic 2-$^{2}H_1$-decanoic acid. ^{1}H NMR analysis of our derivatised product mixture **6** showed that integral of the signal at δ 2.33 (from the 2*S*-proton of the decanoic acid)[14] was much smaller than that of the signal at δ 2.29 (from the 2*R*-proton) (Fig. 2). Comparison of the integrals with those for the OMe signal of the methyl mandelate unit showed that the 2-pro-*S* proton had been replaced approximately five times more often than the 2-pro-*R* proton in the decanoyl-CoA **2**. It is not clear whether this result arises from stereoselective deprotonation of the acyl-CoA substrate or stereoselective reprotonation of the enol/enolate intermediate.

The possibility that binding of straight-chain acyl-CoA substrates could interfere with branched-chain fatty acid metabolism was then investigated. Thus, the catalytic efficiency of proton exchange by AMACR with *S*-2-methyl-decanoyl-CoA **1*S*** and decanoyl-CoA **2** was determined by Michaelis–Menten kinetics. The known substrate *S*-2-methyl-decanoyl-CoA **1*S*** gave the following kinetic parameters: K_m = 614 μM; V_{max} = 88.7 nmol min^{-1} mg^{-1}; k_{cat} = 0.07 s^{-1}; k_{cat}/K_m = 114 M^{-1} s^{-1} at 30 °C, consistent with previous reports.[1] Kinetic analysis of decanoyl-CoA **2** substrate with the AMACR showed non-competitive substrate inhibition at higher substrate concentrations, probably due to incomplete dissolution in the buffer or formation of micelles. The following kinetic parameters were estimated: K_m = 225 μM; V_{max} = 10.6 nmol min^{-1} mg^{-1};

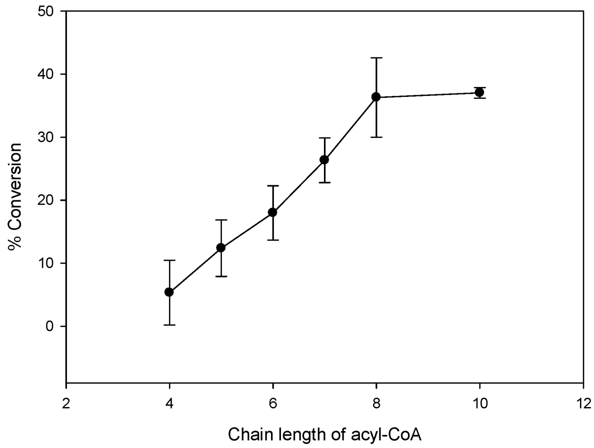


Fig. 3 Exchange of α-[1]H for [2]H in straight-chain acyl-CoAs of differing chain lengths. Error bars are ± one standard deviation (n = 2).

k_{cat} = 0.0084 s^{-1}; k_{cat}/K_m = 37.4 M^{-1} s^{-1}. Therefore, the *S*-2-methyldecanoyl-CoA **1*S*** substrate appears to be exchanged *ca.* 3-fold more efficiently than decanoyl-CoA **2**, as judged by k_{cat}/K_m. However, the non-Michaelis–Menten behaviour of decanoyl-CoA **2** means this difference could be significantly larger. It therefore seems likely that α-proton exchange of straight-chain fatty acyl-CoA esters by AMACR is not physiologically significant in the presence of branched-chain substrates.

The effect of chain-length on the rates of proton exchange of acyl-CoA substrates by AMACR was then investigated by incubation of acyl-CoAs of varying chain lengths with the enzyme for 16 h at 30 °C in buffer containing ^2H_2O. Acetyl-CoA was not significantly converted (≪1% conversion). The extents of exchange of the α-protons for deuterium for C_4–C_{10} straight-chain substrates were measured by ^1H NMR (Fig. 3). Increasing the length of the side-chain of the acyl-CoA ester increased the extent of conversion. Butanoyl-CoA was exchanged to <5%, whilst pentanoyl-CoA, hexanoyl-CoA and heptanoyl-CoA were exchanged to 10–25%. The greatest levels of exchange (*ca.* 40%) were observed for octanoyl-CoA and decanoyl-CoA **2**. Conversion of *S*-2-methyldecanoyl-CoA **1*S*** was >95% under the same conditions. This apparent dependence of the binding and turnover of the substrates on the chain-length is consistent with substrate binding by hydrophobic interactions with the enzyme. Interestingly, the crystal structures of MCR[8] with acyl-CoA ligands bound show that the side-chain of the substrate interacts with a methionine-rich hydrophobic region at the entrance of the active site.

Conversion of 2-methylpropanoyl-CoA (isobutyryl-CoA) by AMACR was also investigated. Only *ca.* 1% exchange of ^1H to ^2H was observed under these assay conditions, compared to <5% for butanoyl-CoA. These two substrates have the same number of carbon atoms and differ only in length of side-chain and in that the former is a branched-chain substrate. Thus, the presence of a methyl group appears to increase the efficiency of catalytic conversion of longer chain substrates (2-methyldecanoyl-CoA **1** *vs.* decanoyl-CoA **2**) but appears to have little effect on catalytic efficiency with short-chain substrates.

In summary, this communication demonstrates that straight-chain fatty acyl-CoA esters are able to bind to recombinant human AMACR 1A and undergo deprotonation and reprotonation events. Only one of the two α-protons is exchanged in each AMACR catalytic cycle and the enzyme has some degree of stereoselectivity. The origin of this stereoselectivity merits further investigation. These results also reveal new details about the catalytic mechanism and substrate binding characteristics of human AMACR 1A. Moreover, this appears to be the only example of a racemase enzyme catalysing proton-exchange in a non-racemisable substrate.

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Notes and references

‡ Abbreviations used: AMACR, α-methylacyl-CoA racemase; MCR, *M. tuberculosis* homologue of AMACR.

1 D. Darley, D. S. Butler, S. J. Prideaux, T. W. Thornton, A. D. Wilson, T. J. Woodman, M. D. Threadgill and M. D. Lloyd, *Org. Biomol. Chem.*, 2009, **7**, 543–552.
2 M. D. Lloyd, D. J. Darley, A. S. Wierzbicki and M. D. Threadgill, *FEBS J.*, 2008, **275**, 1089–1102.
3 A. S. Wierzbicki, M. D. Lloyd, C. J. Schofield, M. D. Feher and F. B. Gibberd, *J. Neurochem.*, 2002, **80**, 727–735.
4 J. A. Mobley, I. Leav, P. Zielie, C. Wotkowitz, J. Evans, Y. W. Lam, B. S. L'Esperance, Z. Jiang and S. Ho, *Cancer Epidemiol. Biomarkers Prev.*, 2003, **12**, 775–783.
5 J. F. Xu, T. Thornburg, A. R. Turner, M. Vitolins, D. Case, J. Shadle, L. Hinson, J. L. Sun, W. N. Liu, B. L. Chang, T. S. Adams, S. L. Zheng and F. M. Torti, *Prostate*, 2005, **63**, 209–214.
6 M. Mukherji, C. J. Schofield, A. S. Wierzbicki, G. A. Jansen, R. J. A. Wanders and M. D. Lloyd, *Prog. Lipid Res.*, 2003, **42**, 359–376.
7 W. Schmitz and E. Conzelmann, *Eur. J. Biochem.*, 1997, **244**, 434–440.
8 P. Bhaumik, W. Schmitz, A. Hassinen, J. K. Hiltunen, E. Conzelmann and R. K. Wierenga, *J. Mol. Biol.*, 2007, **367**, 1145–1161.
9 J. Luo, S. Zha, W. R. Gage, T. A. Dunn, J. L. Hicks, C. J. Bennett, C. N. Ewing, E. A. Platz, S. Ferdinandusse, R. J. Wanders, J. M. Trent, W. B. Isaacs and A. M. De Marzo, *Cancer Res.*, 2002, **62**, 2220–2226.
10 T. Thornburg, A. R. Turner, Y. Q. Chen, M. Vitolins, B. Chang and J. Xu, *Future Oncol.*, 2006, **2**, 213–223.
11 S. Zha, S. Ferdinandusse, S. Denis, R. J. Wanders, C. M. Ewing, J. Luo, A. M. De Marzo and W. B. Isaacs, *Cancer Res.*, 2003, **63**, 7365–7376.
12 K. Takahara, H. Azuma, T. Sakamoto, S. Kiyama, T. Inamoto, N. Ibuki, T. Nishida, H. Nomi, T. Ubai, N. Segawa and Y. Katsuoka, *Anticancer Res.*, 2009, **29**, 2497–2505.
13 A. J. Carnell, I. Hale, S. Denis, R. J. A. Wanders, W. B. Isaacs, B. A. Wilson and S. Ferdinandusse, *J. Med. Chem.*, 2007, **50**, 2700–2707.
14 D. Parker, *J. Chem. Soc., Perkin Trans. 2*, 1983, 83–88.
15 J. M. Schwab and D. C. T. Lin, *J. Am. Chem. Soc.*, 1985, **107**, 6046–6052.

