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

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

Received (in Cambridge, UK) 5th February 2010, Accepted 9th March 2010
First published as an Advance Article on the web 30th March 2010
DOI: 10.1039/c002509g

\( \alpha \)-Methylacyl-CoA racemase (AMACR;† P504S) catalysed exchange of straight-chain fatty acyl-CoA \( \alpha \)-protons. One \( \alpha \)-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 \( \alpha \)-proton exchange 3× more efficiently (as judged by \( K_{cat}/K_m \)) than decanoyl-CoA.

Branched-chain fatty acids, e.g. phytanic acid (3R/S,7R,11R,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 cancer in the male population, 6 and increased levels of phytanic acid in the diet are associated with an increased risk of prostate cancer. 7 The presence of the 3-methyl group in phytic acid prevents \( \beta \)-oxidation, and it is processed as its CoA ester by peroxisomal \( \alpha \)-oxidation to give pristanic acid (2R/S,6R,10R,14-tetramethylpentadecanoic acid). Pristanic acid is metabolised as its CoA ester by \( \beta \)-oxidation in peroxisomes and subsequently in mitochondria for chain-shortened derivatives.2,3,6 The \( \beta \)-oxidation pathway only oxidises \( \alpha \)-methyl fatty acyl-CoA esters with 2S configuration,2,7 but 2R-methylacyl-CoA esters are produced during the degradation of phytanic acid and other endogenous fatty acids. Chiral inversion of these 2R-methylacyl-CoA esters is catalysed by the enzyme \( \alpha \)-methylacyl-CoA racemase (AMACR) (Scheme 1).1,2 and proceeds by removal of an \( \alpha \)-proton to give an enol/enolate intermediate followed by non-stereoselective reprotonation.4 The enzyme has two bases within the active site, the His-122/Glu-237 pair and Asp-156, 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.9 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 \( \alpha \)-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 \( \alpha \)-protons exchanged during each catalytic event? (2) If only one \( \alpha \)-proton is exchanged, is the reaction stereo-selective? (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 \( ^2\)H\(_2\)O. Decanoyl-CoA 2 was chosen as this is the straight-chain analogue of the previously reported \( S \)- and \( R \)-2-methyldecanoyl-CoA substrates (1S and 1R).1 Incubations of 2 with active enzyme resulted in a reduction in the peak intensity of the triplet for the \( \alpha \)-protons at \( \delta \) 2.36–2.46 in the \(^1\)H NMR spectrum, along with changes in the structure of the \( \beta \)-proton quintet at \( \delta \) 1.40. Levels of conversion of ca. 35–40% were observed after incubation for 16 h. Incubation of 2,2-[\( ^2\)H\(_2\)]-decanoyl-CoA 3 (R\(_1\), R\(_2\) = \( ^2\)H) with active AMACR in \( ^3\)H\(_2\)O buffer resulted in the appearance of a triplet in the \(^1\)H NMR spectrum for the \( \alpha \)-protons and changes in the \( \beta \)-protons signal from a triplet into a quintet. High levels of conversion were observed, indicating that the \( \alpha \)-\(^1\)H \( \leftrightarrow \) \( \alpha \)-\(^2\)H exchange reactions are efficiently catalysed by AMACR in both directions. These

\[ R_1 \rightleftharpoons R_2 \]

Scheme 2 Incorporation of deuterium during reaction of straight-chain fatty acyl-CoA esters (decanoyl-CoA 2) with AMACR (R\(_1\), R\(_2\) = \( ^1\)H or \( ^2\)H).
reaction, the CoA ester mixture was hydrolysed and the
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[^13C]-decanoyl-CoA 4 in H2O buffer.
This resulted in exchange of the αH for deuterium, as judged
by 13C NMR spectroscopy. At t = 0, the spectrum of the
incubation mixture showed only a strong singlet at δ 37.65
arising from the 2-13C carrying only 2 × 1H. The 13C 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 2-13C (Fig. 1).
Only at very high levels of conversion was a small quintet
(corresponding to 2-13C2H2) 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 2-13C2H1-decanoyl-
CoA occurs from a second “visit” to the enzyme active site by
2-13C2H1-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 AMACR1,8 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
derprotonated 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
proton on the initial base (to give back an unlabeled acyl-CoA)
only one deuterium was directly attached to the 2-13C (Fig. 1).
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 1H 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 Parker14 who showed that the pro-R 2H 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 2H. Assignments of the more upfield
and more downfield signals are reversed for chiral derivatization
with methyl R-mandelate. Schwab and Lin15 also used this
method to establish the absolute configuration of synthetic
2-3H1-decanoic acid. 1H NMR analysis of our derivatised
product mixture 6 showed that integral of the signal at δ
2.33 (from the 2S-proton of the decanoic acid)14 was much
smaller than that of the signal at δ 2.29 (from the 2R-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 1S and decanoyl-CoA 2 was determined by
Michaelis–Menten kinetics. The known substrate S-2-methyl-
decanoyl-CoA 1S gave the following kinetic parameters:

K_{m} = 614 \mu M; V_{\text{max}} = 88.7 \text{ nmol min}^{-1} \text{ mg}^{-1}; k_{\text{cat}} =
0.07 \text{ s}^{-1}; k_{\text{cat}}/K_{m} = 114 \text{ M}^{-1} \text{ s}^{-1} \text{ at 30 }^\circ \text{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 \mu M; V_{\text{max}} = 10.6 \text{ nmol min}^{-1} \text{ mg}^{-1};

![Fig. 1](image1.png)

![Fig. 2](image2.png)
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For C4–C10 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. Interestingly, the crystal structures of MCR8 with substrate binding by hydrophobic interactions with the entrance of the active site.

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 2H2O. Acetyl-CoA was not significantly converted (≪1% conversion). The extents of exchange of the α-protons for deuterium for C4–C10 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 1S 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 MCR5 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 stereo-selectivity 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.

We thank Mr J. Crossman for purifying human recombinant AMACR 1A. This work was funded by Cancer Research UK and the Higher Education Commission of the Government of Pakistan (support to FAS). Part of this work was undertaken as an Erasmus Exchange Student Project by FP.

### Notes and references