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A continuous assay for α-methylacyl-coenzyme A racemase using circular dichroism

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ABSTRACT

α-Methylacyl-coenzyme A racemase (AMACR) catalyzes the epimerization of (2*R*)- and (2*S*)-methyl branched fatty acyl-coenzyme A (CoA) thioesters. AMACR is a biomarker for prostate cancer and a putative target for the development of therapeutic agents directed against the disease. To facilitate development of AMACR inhibitors, a continuous circular dichroism (CD)-based assay has been developed. The open reading frame encoding AMACR from *Mycobacterium tuberculosis* (MCR) was subcloned into a pET15b vector, and the enzyme was overexpressed and purified using metal ion affinity chromatography. The rates of MCR-catalyzed epimerization of either (2*R*)- or (2*S*)-ibuprofenoyl-CoA were determined by following the change in ellipticity at 279 nm in the presence of octyl-β-D-glucopyranoside (0.2%). MCR exhibited slightly higher affinity for (2*R*)-ibuprofenoyl-CoA ($K_m = 48 \pm 5$ μM, $k_{cat} = 291 \pm 30$ s^{-1}), but turned over (2*S*)-ibuprofenoyl-CoA ($K_m = 86 \pm 6$ μM, $k_{cat} = 450 \pm 14$ s^{-1}) slightly faster. MCR expressed as a fusion protein bearing an N-terminal His_6-tag had a catalytic efficiency (k_{cat}/K_m) that was reduced 22% and 47% in the 2*S* → 2*R* and 2*R* → 2*S* directions, respectively, relative to untagged enzyme. The continuous CD-based assay offers an economical and efficient alternative method to the labor-intensive, fixed-time assays currently used to measure AMACR activity.

α-Methylacyl-coenzyme A racemase (AMACR,[1] EC 5.1.99.4, also known as P504S) catalyzes the 1,1-proton transfer reaction [1,2] that effects the reversible epimerization of the coenzyme A (CoA) thioesters derived from a variety of (2*R*)- and (2*S*)-methyl branched fatty acids [3], including pristanic acid and its chain-shortened derivatives, a number of C_{27} bile acid intermediates [4], and 2-arylpropionic acids (e.g., ibuprofen) [5,6]. Studies using 2-^{2}H- or 2-^{3}H-labeled substrates and the rat [3,7], human [2,8], and *Mycobacterium tuberculosis* [1,9] enzymes suggest that the reaction proceeds via an enol/enolate intermediate. This cofactor-independent enzyme is localized in mitochondria and peroxisomes [10], and it is in the latter organelle where only α-methylacyl-CoAs with the (2*S*)-stereochemistry are metabolized [11]. Because pristanic acid and its precursors contain (2*R*)-methyl groups, AMACR-catalyzed epimerization permits metabolites derived from phytanic acid (originating from chlorophyll catabolism) to enter into the peroxisomal β-oxidation pathway [12].

Elevated levels of AMACR have been associated with various cancers [13–15], and the enzyme serves as a biomarker for prostate cancer (PCa) [16–18]. Interestingly, the peroxisomal branched chain fatty acid β-oxidation pathway has particular relevance to PCa: (i) the pathway is up-regulated in PCa and appears to serve as a major source of energy [19,20]; (ii) peroxisomal β-oxidation generates H_2O_2, a potential source of oxidative damage [21–23]; and (iii) the main sources of branched chain fatty acids in humans (i.e., phytanic acid arising from milk, beef, and dairy products [24]) have been implicated as risk factors for developing PCa [25,26]. This suggests that inhibition of metabolic flux through the β-oxidation pathway could slow progression of PCa. Interestingly, epidemiological studies have revealed that ingestion of nonsteroidal antiinflammatory drugs such as ibuprofen reduces the risk for developing PCa [27], perhaps by competing for the active site of AMACR with other fatty acyl-CoA thioesters required for energy. Recently, Takahara and coworkers [28] showed that inhibition of AMACR expression using small interfering RNA (siRNA) induced an increase in the expression of androgen receptor accompanied by a decrease in the expression of genes associated with cancer progression, including insulin-like growth factor I and platelet-derived growth factor alpha in the C4-2 PCa cell line. Hence, AMACR

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[1] *Abbreviations used:* AMACR, α-methylacyl-coenzyme A racemase; CoA, coenzyme A; PCa, prostate cancer; siRNA, small interfering RNA; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; CD, circular dichroism; MCR, AMACR homologue from *M. tuberculosis;* MeCN, acetonitrile; THF, tetrahydrofuran; SPE, solid-phase extraction; TLC, thin layer chromatography; UV, ultraviolet; ORF, open reading frame; PCR, polymerase chain reaction; His_6, hexahistidine; LB, Luria–Bertani; IPTG, isopropyl β-D-1-thiogalactopyranoside; His_6–MCR, MCR with an N-terminal hexahistidine tag; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EGTA, ethyleneglycoltetraacetic acid; OG, octyl-β-D-glucopyranoside.

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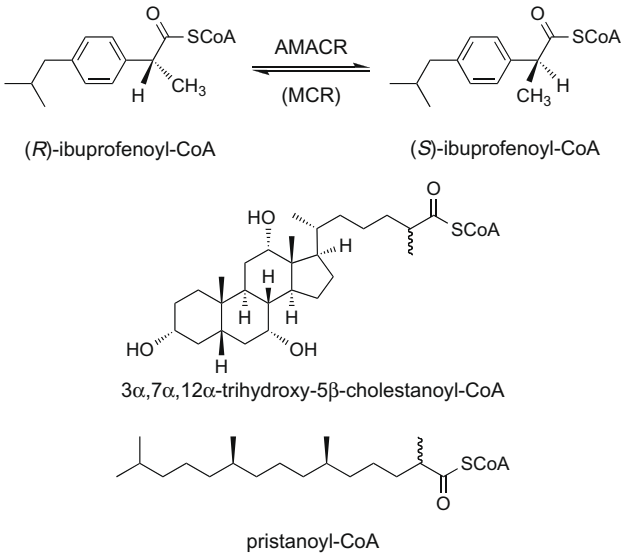
inhibition may be useful for treatment of patients with hormone refractory prostate cancer. Inhibition of AMACR activity may also act synergistically with androgen ablation therapy [19].

To date, the effort to develop inhibitors of AMACR has been somewhat limited [29]. The screening of compounds as potential inhibitors requires the development of a rapid and convenient assay of AMACR activity. Currently, labor-intensive, fixed-time assays for determining AMACR activity are employed. One assay involves termination of the AMACR-catalyzed reaction with HCl followed by heating to hydrolyze the CoA thioesters and subsequent conversion of the resulting (*R*)- and (*S*)-free acids to their amides with (*R*)-1-phenylethylamine. These diastereomeric amides are then analyzed using gas–liquid chromatography (GLC) [3]. Alternatively, the resulting (*R*)- and (*S*)-free acids have been derivatized with (1*R*,2*S*,5*R*)-(−)-menthol to yield the corresponding diastereomeric menthyl esters that were analyzed using reversed-phase high-performance liquid chromatography (HPLC) [5]. Another assay method involves the use of either [2-^{3}H]pristanoyl-CoA or [24,25-^{3}H]3α,7α,12α-trihydroxy-5β-cholestanoyl-CoA as the substrate. The AMACR-catalyzed reactions are terminated by the addition of trichloroacetic acid, the substrate is adsorbed on a reversed-phase matrix, and the ^3H_2O resulting from the enzyme-catalyzed deprotonation of the substrates and exchange with solvent H_2O is quantified using scintillation counting [3,9]. Recently, the kinetic parameters for human AMACR with (*R*)- and (*S*)-2-methyltetradecanoyl-CoA as the substrates were determined using a nuclear magnetic resonance (NMR)-based method [2]. AMACR activity was measured by following the exchange of the substrate α-proton with solvent D_2O using ^{1}H NMR spectroscopy after quenching the reaction with NaOH. Similar NMR-based assays have also been employed using AMACR from rat [3] and *M. tuberculosis* [1,9]. These fixed-time assays may have the added complication of a solvent isotope effect.

The current fixed-time assays for determining AMACR activity have several major disadvantages, including the need to terminate the reaction (and often to remove protein), the need to prepare derivatives of either the substrates or the products, and the length of time required for the execution of various separation techniques. Here we report a continuous circular dichroism (CD)-based assay for measuring AMACR activity. Using the AMACR homologue from *M. tuberculosis* (MCR), which is 43% identical to human AMACR [9], and either (2*R*)- or (2*S*)-ibuprofenoyl-CoA as the substrate, we show that the CD-based assay offers a quick, inexpensive, and effective method for monitoring AMACR activity (Scheme 1).



Scheme 1. Some substrates for AMACR include (*R*)- and (*S*)-ibuprofenoyl-CoA, 3α,7α,12α-trihydroxy-5β-cholestanoyl-CoA, and pristanoyl-CoA.

Materials and methods

Racemic ibuprofen sodium salt, 1,1′-carbonyldiimidazole, and (*R*)-(+)-α-methylbenzylamine were purchased from Sigma–Aldrich Canada (Oakville, ON, Canada). (*S*)-Ibuprofen was purchased from Fluka Analytical (Buchs, Switzerland). CoA (trilithium salt) was purchased from BioShop Canada (Burlington, ON, Canada). *M. tuberculosis* genomic DNA was obtained from J. T. Belisle (University of Colorado, produced under National Institutes of Health/National Institute of Allergies and Infectious Diseases contract HHSN266200400091C/ADB contract NO1-AI-75320, "Tuberculosis Vaccine Testing and Research Materials Contract"). Acetonitrile (MeCN, HPLC grade) was purchased from Fischer Scientific (Ottawa, ON, Canada). Deoxyoligonucleotide primers were commercially synthesized by ID Labs (London, ON, Canada). Restriction enzymes were purchased from New England Biolabs (Pickering, ON, Canada). His·Bind resin and thrombin cleavage capture kits were purchased from Novagen (Madison, WI, USA), and *Pfu Turbo* DNA polymerase was purchased from Stratagene (La Jolla, CA, USA). All other chemicals were reagent grade or better. Tetrahydrofuran (THF) was dried and distilled over sodium. Melting points (uncorrected) were determined using an Electrothermal Melt-Temp model 1201D capillary melting point apparatus (Barnstead International, Dubuque, IA, USA). Optical rotations were measured using a Rudolph Instruments Digi 781 automatic polarimeter (Denville, NJ, USA).

Resolution of (R)-ibuprofen

(*R*)-Ibuprofen was resolved using the protocol described by Trung and coworkers [30] with modifications. Aqueous HCl (1 M) was added to a solution of racemic ibuprofen (20 g, sodium salt) dissolved in water (100 ml) until the pH was 2.0. The free acid was then obtained after extraction into diethyl ether and removal of the solvent under reduced pressure. The racemic free acid (4.0 g, 19.2 mmol) was then dissolved in absolute ethanol (48 ml). (*R*)-(+)-α-Methylbenzylamine (2.5 ml, 19.2 mmol) was added at 60 °C with stirring, and the resulting solution was refluxed for 15 min. Upon cooling to room temperature, white crystals formed which were isolated by filtration. After three successive recrystallizations from ethanol, the diastereomeric salt (1.0 g) was dissolved in 1 M HCl (50 ml) to liberate the free acid of (*R*)-ibuprofen. Extraction of the aqueous solution with diethyl ether followed by removal of the solvent under reduced pressure afforded (*R*)-ibuprofen (0.43 g) as a white powder, melting point 50–52 °C (48–49 °C [31]), $[\alpha]_D^{20}$ −(56.08 ± 0.02)° (*c* = 2.5, ethanol) (lit. $[\alpha]_D^{25}$ −53° (*c* = 2, ethanol) [31]).

Ibuprofenoyl-CoA

Synthesis of ibuprofenoyl-CoA was conducted using the protocol described by Sidenius and coworkers [32] with minor modifications. 1,1′-Carbonyldiimidazole (0.0324 g, 200 μmol), dissolved in anhydrous THF (1 ml), was added to a solution of ibuprofen free acid (0.0206 g, 100 μmol) in anhydrous THF (1 ml). The mixture was stirred at room temperature under an argon atmosphere for 2 h. Water (0.5 ml) was then added, followed by the trilithium salt of CoA (0.0411 g, 50 μmol) dissolved in water (0.5 ml). After 24 h, the reaction was quenched by the addition of 1 M HCl until the pH of the solution was 2.0. Unreacted ibuprofen was removed from