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Human mitochondrial amidoxime reducing component (mARC): An electrochemical method for identifying new substrates and inhibitors

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ABSTRACT

As recently as 2006 the mitochondrial amidoxime reducing component (mARC) was identified as the fourth and last Mo enzyme present in humans. Its physiological role remains unknown. mARC is capable of reducing a variety of *N*-hydroxylated compounds such as amidoximes to their corresponding amidine and there is considerable interest in this enzyme from a pharmaceutical perspective. mARC is a target for *N*-hydroxylated prodrugs that may be reductively activated intracellularly to release potent drugs such as cationic amidinium ions, which exhibit a broad spectrum of activity as antithrombotics and against various bacteria and parasites. In the quest for a rapid screen of new mARC substrates and inhibitors we present an electrochemical method which utilizes the natural electron partner of mARC, cytochrome b_5, coupled to an electrochemical electrode. Mediated electron transfer from the electrode via cytochrome b_5 to mARC results in a catalytic current in the presence of substrate.

1. Introduction

In 2006, the mitochondrial amidoxime reducing component (mARC) was the fourth, and last, Mo-dependent enzyme identified in humans [1]. Like the well-studied human sulfite oxidase, mARC is also located in mitochondria, while the remaining two Mo enzymes (xanthine oxidase and aldehyde oxidase) are found in the cytosol [2]. In addition to its mitochondrial localization, mammalian mARC has been detected in peroxisomes as well [3]. Although no crystal structure of a mARC enzyme has been reported, sequence analysis and spectroscopy have shown that it belongs in the sulfite oxidase family [4] according to the enduring Mo enzyme classification originally proposed by Hille [5] (Scheme 1).

The 35 kDa mARC enzyme bears no cofactors other than the Mo active site (Scheme 1) and, in its reduced Mo^{IV} form, catalyses the reduction of *N*-hydroxylated compounds including, but not limited to, amidoximes and hydroxylamines to their corresponding amidines or amines [4]. mARC is found in two isoforms (mARC1 and mARC2), which share 80% sequence similarity [6], but, depending on the species and tissue, only one mARC protein is expressed predominately [7,8]. The physiological function of both mARC1 and mARC2 in humans and other eukaryotes is still unknown. The enzymes appear to be involved in metabolic detoxification reactions [7,8], the NO pathway by aerobic reduction of the NO-precursor *N*ω-hydroxy-L-arginine or anaerobic reduction of nitrite to NO. [9] Moreover, mARC is implicated in energy and lipid metabolism as well as metabolic disorders as diabetes mellitus [4,10].

There is much interest in human mARC from the perspective of drug metabolism. By analogy with the cytochromes P450, which frequently hydroxylate and deactivate xenobiotic drug-like compounds, mARC is able to reduce *N*-hydroxylated compounds in a complementary way. This may be turned to advantage in drug design [11] through the administration of *N*-hydroxylated prodrugs which, after reductive de-hydroxylation in vivo by mARC, can release active drugs already delivered to the target cell. This is particularly useful for very active amidines (Scheme 2), which, due to their high basicity, are protonated at physiological pH (to give the amidinium conjugate acid) and unable to cross the cell membrane.

However, in their charge-neutral amidoxime form they are sufficiently hydrophobic to penetrate the cell by passive diffusion and act as a 'Trojan horse' that is activated intracellularly by mARC catalyzed reduction. In other cases toxic *N*-hydroxylated metabolites formed by P450 enzymes are reduced by mARC to their corresponding dehydroxylated parent compound. Due to its involvement in many

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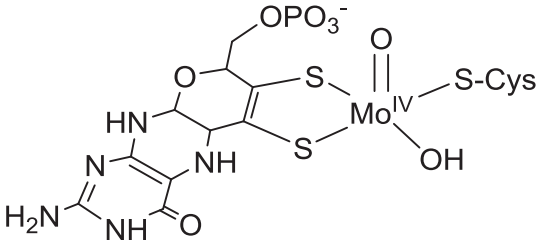
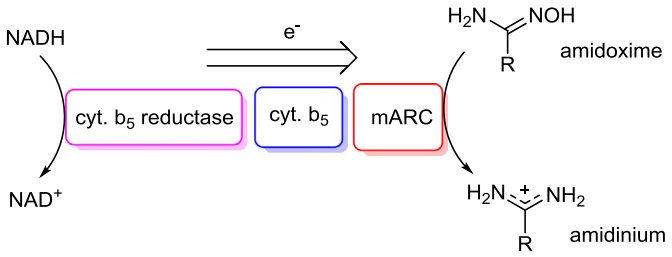


Scheme 1. Active site of mARC in its Mo^{IV} form (overall charge of complex ion not shown).



Scheme 2. Electron flow during mARC catalysis.

activation, deactivation and detoxification reactions, mARC is a new and essential addition to the list of known drug metabolizing enzymes [11].

The overall electron transfer sequence for mARC catalysis is illustrated in Scheme 2. Reducing equivalents supplied by NADH are passed to the flavoprotein cytochrome b_5 reductase, then, one at a time, to the heme protein cytochrome b_5 before being relayed to mARC to prime the Mo^{IV} active site for substrate reduction [8]. The natural substrate (or substrates) for mARC1 and mARC2 remain unknown. This is complicated by the fact that mARC is able to reduce a variety of organic compounds with N-O functionality [4].

Being an Mo-dependent oxidoreductase, mARC may be examined by electrochemistry [12] and that is the focus of this investigation. In the quest for a rapid and high throughput assay of potential drug-like mARC substrates and inhibitors, we present a new cyclic voltammetry (CV) methodology that utilizes miniscule (picomole) quantities of enzyme and potentially may screen many new drug candidates rapidly and efficiently.

2. Materials and methods

A gold working electrode (BAS Inc.) was chemically modified with the bifunctional thiol 3-mercaptosuccinic acid (MSA) following a literature protocol [13]. A Pt wire counter electrode and Ag/AgCl reference electrode were incorporated into a BAS C3 cell stand and attached to a BAS100 potentiostat. All electrochemical experiments were carried out under an atmosphere of dinitrogen. Human mARC1 and cytochrome b_5 were recombinantly expressed in *E. coli* and purified as described [6]. A mixture of 4 μL of recombinant human cyt.b_5 (17 μM) and 2 μL of 0.25% chitosan solution (in 1% acetic acid) was pipetted onto the conducting surface of an inverted, freshly prepared Au/MSA working electrode and allowed to evaporate to a film at 4 °C. After that 2 μL of mARC1 (250 μM) was dispensed onto the same Au/MSA/chitosan-cyt.b_5 electrode and again allowed to evaporate over 1 h at 4 °C to a thin film. To prevent protein loss the enzyme modified electrode surface was carefully covered with a perm-selective dialysis membrane (molecular weight cut off 3500 Da), pre-soaked in water. The dialysis membrane was pressed onto the electrode with a Teflon cap and fastened to the electrode with a rubber O-ring to prevent leakage of the internal membrane solution. The resulting enzyme modified electrode was stored at 4 °C in 100 mM phosphate buffer (pH 6.0) when not in use. The proteins cyt. b_5 and mARC were confined to a thin layer beneath the membrane with chitosan while the substrates were able to diffuse across the membrane. Phosphate buffer (100 mM) was used for experiments at pH 6. For pH-dependent experiment, the mixture of buffers (25 mM citric acid buffer pH 3.0–6.2, 25 mM Bis-Tris buffer pH 5.8–7.2, 25 mM Tris buffer pH 7.0–9.0 and 25 mM CHES buffer pH 8.6–10.0) were used and the desired pH was obtained by titration with dilute acetic acid or NaOH. All solutions were prepared with ultrapure water (resistivity 18.2 MΩ·cm).

3. Results and discussion

We employed the native mARC1 partner, outer mitochondrial membrane cytochrome b_5 (cyt. b_5), as a meditator of electron transfer between a chemically modified Au working electrode and the enzyme. This enables catalytic electrochemistry to take place at the relatively high potential of the cyt. b_5 redox couple thus avoiding non-specific reduction of interfering species at lower potentials. The working electrode replaces NADH and cyt. b_5 reductase in Scheme 2.

UV–vis monitored spectroelectrochemistry of cyt. b_5 was conducted at pH 8 using a published set of electron transfer mediators [14,15] (see Supporting Information Fig. S1 for details) yielding a $Fe^{III/II}$ redox potential of − 50 (± 5 mV) vs SHE which is comparable with previous investigations of human and other vertebrate cyt. b_5 proteins [16–18].

Cyclic voltammetry of cyt. b_5 (Fig. 1) was achieved using a gold working electrode chemically modified with a self-assembled monolayer (SAM) of mercaptosuccinic acid (MSA). As the overall goal was electrochemically driven catalysis of mARC1, we trapped both cyt. b_5 and mARC1 under a semi-permeable membrane (MW cutoff 3.5 kDa) which enabled small volumes (microliters) of protein solution (micromolar concentration) to be employed. An optimal cyt. b_5 response was obtained with the promoter chitosan (poly-D-glucosamine) also present. Chitosan is electro-inactive but greatly enhances the cyt. b_5 current response without altering the redox potential relative to experiments carried out in its absence (Supporting Information Fig. S2). The protonation constants of the glucosamine monomers within chitosan lie in the range pK_a 6.5–7 [19,20] so at all pH values investigated here chitosan bears a net positive charge.

A stable and reversible ferric/ferrous cyt. b_5 voltammetric response is apparent in Fig. 1 within the range 4.56 < pH < 6.55 and the redox potential is almost pH independent (− 1 mV at pH 4.56 and − 19 mV vs SHE at pH 6.55). It has been noted previously that the

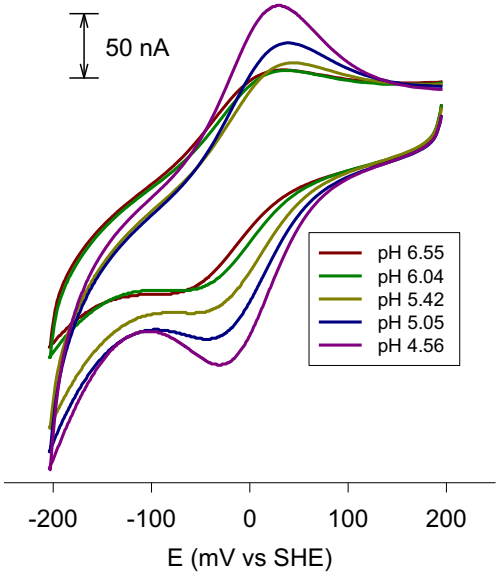


Fig. 1. CVs obtained at a Au/MSA/chitosan-cyt.b_5/mARC1 electrode at various pH values (0.1 M mixed buffer solution titrated with AcOH/NaOH) and a scan rate of 5 $mV^{\circ}s^{-1}$.