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The Mitochondrial Amidoxime Reducing Component (mARC) Is Involved in Detoxification of N-Hydroxylated Base Analogues

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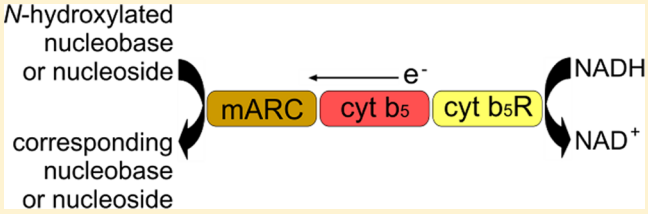
Supporting Information

ABSTRACT: The "mitochondrial Amidoxime Reducing Component" (mARC) is the newly discovered fourth molybdenum enzyme in mammals. All hitherto analyzed mammals express two mARC proteins, referred to as mARC1 and mARC2. Together with their electron transport proteins cytochrome b_5 and NADH cytochrome b_5 reductase, they form a three-component enzyme system and catalyze the reduction of N-hydroxylated prodrugs. Here, we demonstrate the reductive detoxification of toxic and mutagenic N-hydroxylated nucleobases and their corresponding nucleosides by the mammalian mARC-containing enzyme system. The N-reductive activity was found in all tested tissues with the highest detectable conversion rates in liver, kidney, thyroid, and pancreas. According to the presumed localization, the N-reductive activity is most pronounced in enriched mitochondrial fractions. In vitro assays with the respective recombinant three-component enzyme system show that both mARC isoforms are able to reduce N-hydroxylated base analogues, with mARC1 representing the more efficient isoform. On the basis of the high specific activities with N-hydroxylated base analogues relative to other N-hydroxylated substrates, our data suggest that mARC proteins might be involved in protecting cellular DNA from misincorporation of toxic N-hydroxylated base analogues during replication by converting them to the correct purine or pyrimidine bases, respectively.



INTRODUCTION

Until a few years ago, humans were assumed to possess only three molybdenum-containing enzymes, that is, sulfite oxidase, aldehyde oxidase, and xanthine oxidoreductase.[1−3] Each of these proteins is characterized by the essential need for the so-called molybdenum cofactor (Moco), whose molybdenum atom is coordinated to a 5,6,7,8-tetrahydropyranopterin.[4] Recently, a hitherto unknown Moco-containing enzyme was purified from the outer membrane of pig liver mitochondria.[5] The new MOSC-containing (Moco sulfurase C-terminal domain)[6] protein has been found to function in concert with the electron transport proteins NADH cytochrome b_5 reductase (cyt b_5R) and cytochrome b_5 (cyt b_5), thereby forming a redox chain that catalyzes the reduction of N-hydroxylated compounds such as amidoximes, which are often used as prodrugs.[5,7−9] According to its subcellular localization and its reactivity toward amidoximes the MOSC protein was renamed into "mitochondrial Amidoxime Reducing Component" (mARC).[5] Only recently, Neve and co-workers have demonstrated that the mARC-mediated N-reductive pathway might play an important role in lipid synthesis in adipocyte mitochondria.[10] All fully sequenced and annotated mammalian genomes code for two mARC proteins, which are referred to as mARC1 and mARC2 (formerly known as MOSC1 and MOSC2).[9]

In all mammals, the respective mARC isoforms share a high degree of sequence similarity, and after reconstitution with cyt b_5 and cyt b_5R, both enzymes catalyze N-reductive reactions in vitro with equal specificity for some substrates but different specificities for others.[9,11] The involvement of the mARC-containing enzyme system in N-reductive drug metabolism is well accepted,[7,12] and it is assumed that the N-reductive enzyme system plays a major role in the oxygen-independent reduction of N-hydroxylated compounds. Therefore, we speculate that the mARC-containing enzyme system is also involved in the detoxification of mutagenic and toxic N-hydroxylated aromatic amines such as N-hydroxylated nucleobases and nucleosides.[13] The latter compounds are produced as metabolites within physiological cellular metabolism by the action of cytochrome P450 enzymes.[14] In addition, chemical factors such as hydroxylamines can be involved in these transformations. When adenine is exposed to peroxy-radicals, the majority of the newly formed oxidation products is N^6-hydroxyadenine, a potent mutagenic base analogue.[15] Such N-hydroxylated base analogues were shown to be converted into the deoxynucleoside triphosphates and incorporated into replicating DNA.[16] Because of their ambivalent base-pairing

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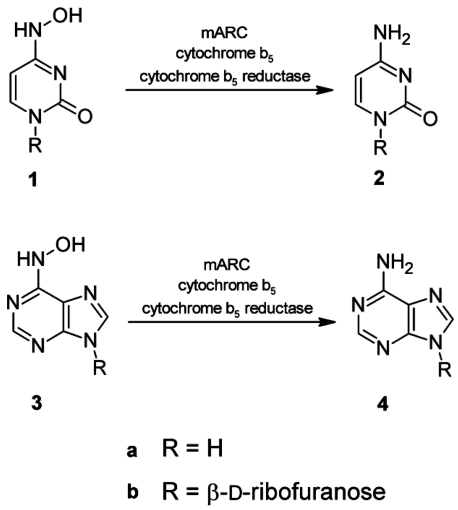
capacity, incorporation of these base analogues induces mutations or DNA strand breaks.[16−18]

In the present study, the reduction of the mutagenic N-hydroxylated nucleobases N^4-hydroxycytosine and N^6-hydroxyadenine as well as the nucleosides N^4-hydroxycytidine and N^6-hydroxyadenosine by the mARC-containing enzyme system was investigated. For precise quantification of the N-hydroxylated base analogues and their corresponding metabolites, HPLC-based analytics were developed. The reductive detoxification of the nucleobases was characterized using subcellular fractions from different porcine tissues and human recombinant enzyme systems (Scheme 1). The results indeed showed that both the native mARC-containing fractions and the recombinant enzyme system efficiently catalyze the detoxification of N-hydroxylated base analogues.

Scheme 1. Reduction of N-Hydroxylated Nucleobases and Nucleosides by the mARC-Containing Three-Component Enzyme System[a]



[a] N^4-hydroxycytosine (**1a**), cytosine (**2a**); N^4-hydroxycytidine (**1b**), cytidine (**2b**); N^6-hydroxyadenine (**3a**), adenine (**4a**); and N^6-hydroxyadenosine (**3b**), adenosine (**4b**).

EXPERIMENTAL PROCEDURES

Chemicals. N^4-hydroxycytosine was purchased from R. I. Chemicals (Orange, United States). N^4-hydroxycytidine was kindly supplied by Dr. Schinazi (Veterans Affairs Medical Center, Decatur, United States). N^6-hydroxyadenine was purchased from MP Biomedicals (Solon, United States). N^6-hydroxyadenosine was synthesized as described before.[19] All N-hydroxylated compounds were >97% pure as determined by HPLC analysis. Traces of the corresponding bases and nucleosides were observed, quantified, and taken into account for the calculation of metabolic rates (see below). All other chemicals were purchased from Sigma-Aldrich (Munich, Germany), Merck KGaA (Darmstadt, Germany), or Roth (Karlsruhe, Germany) unless otherwise stated. Methanol was of HPLC grade and purchased from J. T. Baker (Derventer, Holland).

Preparation of Subcellular Fractions. Porcine tissue was fractionated into homogenate, mitochondria, cytosol, and microsomes. Mitochondria were prepared by gradient centrifugation as described by Hovius et al.[20] with slight modifications. Microsomes were separated from the cytosol by centrifugation at 140000*g*.

Expression and Purification of Recombinant Human Proteins. Expression and purification of mARC1 (reference sequence NP_073583), mARC2 (reference sequence NP_060368), mitochondrial cyt b_5 (reference sequence NP_085056), and cyt b_5R isoform 2 (reference sequence NP_015565) were performed according to Wahl et al.[9]

SDS-PAGE and Immunoblot Analysis. SDS-PAGE was carried out using a separation gel containing 12.5% polyacrylamide according to a method of Laemmli.[21] The protein samples were pretreated with β-mercaptoethanol for 5 min at 100 °C prior to loading onto the gel. Separated proteins were blotted on a polyvinylidinefluoride transfer membrane. For immunoblot analysis, the primary antibodies anti-MOSC1 (Abgent, San Diego, CA), anti-MOSC2 (Santa Cruz Biotechnology, Heidelberg, Germany, or Sigma-Aldrich), anti-GAPDH (glycerinaldehyde-3-phosphate dehydrogenase) (Sigma-Aldrich), anti-Calnexin (Acris antibodies, Herford, Germany), and anti-VDAC (voltage-dependent anion channel) (Acris antibodies) were used. Visualization was done with secondary horseradish peroxidase-conjugated antirabbit IgG (immunoglobuline G) or antigoat IgG antibodies (Jackson Immuno Research Laboratories, Suffolk, United Kingdom), and detection of the chemiluminescence was carried out by using the ECL (enhanced chemiluminescence) Plus Western Blotting Detection System (GE Healthcare, United Kingdom) according to the manufacturer's instructions.

Determination of Protein Concentration. For the determination of the protein content, a BCA (bicinchoninic acid) Protein Assay Kit (Pierce, Rockford, United States) was used according to the manufacturer's instructions.

Determination of Mo by Inductively Coupled Mass Spectrometry (ICP-MS). Molybdenum contents of recombinant human mARC1 and mARC2 proteins were determined by using ICP-MS as described by Kotthaus et al.[8] Measurements were carried out in triplicates.

Flavin Adenine Dinucleotide (FAD) Content Determination. To determine the FAD content of cyt b_5R, samples were heated for 10 min at 100 °C. After centrifugation, the supernatant was analyzed at 450 nm.[22] Measurements were carried out in duplicates.

Heme Content Determination. The heme content was estimated by recording the spectrum of NADH-reduced protein subtracted by the spectrum of oxidized protein.[23] Measurements were carried out in triplicates.

Determination of mARC-Catalyzed N-Reduction. Incubations were carried out under aerobic conditions at 37 °C in a shaking water bath. Incubation mixtures contained different substrate concentrations as indicated, 1.0 mM NADH in a total volume of 150 μL of 20 mM 2-morpholinoethanesulfonic acid (MES) buffer, pH 6.0 (in case of recombinant protein), or 100 mM potassium phosphate buffer, pH 6.0 (in case of subcellular fractions). After 3 min of preincubation at 37 °C, reactions were initiated by addition of NADH and terminated after 15 (recombinant proteins) or 20 min (subcellular fractions) by addition of 150 μL of ice-cold methanol. Precipitated proteins were separated by centrifugation, and the supernatant was analyzed by HPLC. Incubations were carried out in duplicates. Incubation mixtures with recombinant enzymes were adjusted based on their cofactors and consisted of 76 pmol of heme (cyt b_5), 7.6 pmol of FAD (cyt b_5R), and 76 pmol of Mo (mARC1 or mARC2, respectively). Incubation mixtures with subcellular fractions contained 0.01−0.1 mg of protein. Apparent kinetic parameters K_m and V_{max} were estimated using nonlinear regression analysis (Sigma Plot 11.0; SPSS Science, Chicago, IL). For the calculation of kinetic parameters of mARC1 and porcine liver mitochondria with N^6-hydroxyadenine, the linear plot of Lineweaver−Burk in the range between 0.1 and 1.0 mM was used.

HPLC Analysis. The HPLC system consisted of a controller 600 from Waters (Milford, United States), equipped with an autosampler (Waters, 717 plus) and a variable wavelength UV detector (Waters 2487 Dual λ Absorbance Detector). Peak areas were integrated using the EZChrom Chromatography Data System (EZChrom Elite Version 2.8.3, Scientific Software Inc., San Ramon, United States). Separations were carried out isocratically at a flow rate of 1.0 mL/min. The injected sample volume was 10 μL. Aqueous solvents were filtered through a Sartorius polyamide membrane filter (0.45 μm, Sartorius AG, Goettingen, Germany), and organic solvents were of HPLC grade. The mobile phase was degassed by sonication.

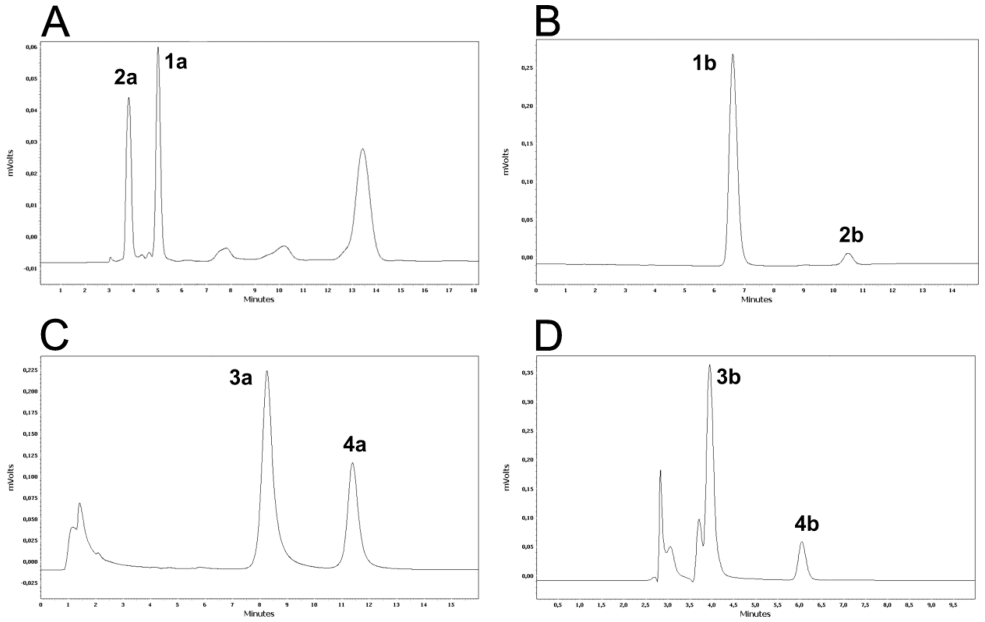


Figure 1. HPLC analytic of modified bases and their reduced metabolites. Representative chromatograms of incubation mixtures with heat-denaturated protein, which were spiked with N-hydroxylated base analogue and its metabolite, are shown. (A) Separation of cytosine (**2a**) and N^4-hydroxycytosine (**1a**), (B) separation of N^4-hydroxycytidine (**1b**) and cytidine (**2b**), (C) separation of N^6-hydroxyadenine (**3a**) and adenine (**4a**), and (D) separation of N^6-hydroxyadenosine (**3b**) and adenosine (**4b**). The chromatographic parameters are described in detail in the Experimental Procedures.

HPLC Method for the Separation of N^4-Hydroxycytosine and Cytosine. Separations were carried out with 95% (v/v) 20 mM ammonium acetate buffer and 5% (v/v) methanol using a Nucleodur 100-5 C18 ec, 4.6 mm × 250 mm (Macherey-Nagel, Dueren, Germany) with a security guard cartridge system C18, 3 mm × 4 mm (Phenomenex, Torrance, CA) as the precolumn. The effluent was monitored at 267 nm. The recovery rate and quantification limit of the metabolite cytosine were determined by incubation mixtures with defined concentrations of synthetic reference substance (from 5 to 500 μM), which were incubated and worked up under the same conditions as the experimental samples using heat-denaturated protein. The signals (peak areas) obtained were compared with those of the same amount of cytosine dissolved in buffer/methanol. The standard curves were linear in this range with regression coefficients of 0.99996 (n = 28). The recovery rate after incubation and sample workup was 102 ± 11% after subtraction of minor contaminations of cytosine in the substrate (2.2%). The retention times were 5.0 ± 0.1 (N^4-hydroxycytosine) and 3.9 ± 0.1 min (cytosine).

HPLC Method for the Separation of N^4-Hydroxycytidine and Cytidine. Separations were carried out with 10 mM 1-octylsulfonate sodium salt [75% (v/v)], pH 2.0, and methanol [25% (v/v)] using a LiChrospher 60 RP-select B column (250 mm × 4 mm, 5 μm, Merck KGaA) with a RP select B guard precolumn (4 mm × 4 mm, Merck KGaA). The effluent was monitored at 281 nm. The recovery rate and quantification limit of the metabolite cytidine were determined by incubation mixtures with defined concentrations of synthetic reference substance (from 5 to 500 μM), which were incubated and worked up under the same conditions as the experimental samples using heat-denaturated protein. The signals (peak areas) obtained were compared with those of the same amount of cytidine dissolved in buffer/methanol. The standard curves were linear in this range with regression coefficients of 0.9997 (n = 36). The recovery rate after incubation and sample workup was 108 ± 8%. The retention times were 5.9 ± 0.1 (N^4-hydroxycytidine) and 9.5 ± 0.1 min (cytidine).

HPLC Method for the Separation of N^6-Hydroxyadenine and Adenine. Separations were carried out with 20 mM 1-octylsulfonate sodium salt [82% (v/v)], pH 3.0, and methanol [18% (v/v)] using a LiChrospher 60 RP-select B column (125 mm × 4 mm, 5 μm, Merck KGaA) with a RP select B guard precolumn (4 mm × 4 mm, Merck KGaA). The effluent was monitored at 262 nm. The recovery rate and quantification limit of the metabolite adenine were determined by incubation mixtures with defined concentrations of synthetic reference substance (from 10 to 500 μM), which were incubated and worked up under the same conditions as the experimental samples using heat-denaturated protein. The signals (peak areas) obtained were compared with those of the same amount of adenine dissolved in buffer/methanol. The standard curves were linear in this range with regression coefficients of 0.9998 (n = 36). The recovery rate after incubation and sample workup was 106 ± 11% after subtraction of minor contaminations of adenine in the substrate (0.8%). The retention times were 8.3 ± 0.1 (N^6-hydroxyadenine) and 11.5 ± 0.1 min (adenine).

HPLC Method for the Separation of N^6-Hydroxyadenosine and Adenosine. Separations were carried out with 20 mM ammonium acetate buffer [80% (v/v)] and methanol [20% (v/v)] using a Nucleodur 100-5 C18 ec, 4.6 mm × 250 mm (Macherey-Nagel, Dueren, Germany) with a security guard cartridge system C18, 3 mm × 4 mm (Phenomenex) as the precolumn. The effluent was monitored at 260 nm. The recovery rate and quantification limit of the metabolite adenosine were determined by incubation mixtures with defined concentrations of synthetic reference substance (from 1 to 500 μM), which were incubated and worked up under the same conditions as the experimental samples using heat-denaturated protein. The signals (peak areas) obtained were compared with those of the same amount of adenosine dissolved in buffer/methanol. The standard curves were linear in this range with regression coefficients of 0.9999 (n = 36). The recovery rate after incubation and sample workup was 114 ± 8% after subtraction of minor contaminations of adenosine in the substrate (0.1%). The retention times were 4.1 ± 0.1 (N^6-hydroxyadenosine) and 6.4 ± 0.1 min (adenosine).

RESULTS

HPLC Methods for Separation of Nucleobases and Nucleosides from their Corresponding N-Hydroxylated Analogues. In this study, HPLC methods for the separation and quantification of N-hydroxylated nucleobases and nucleosides from their reduced metabolites have been developed. These methods are characterized by an accurate quantification

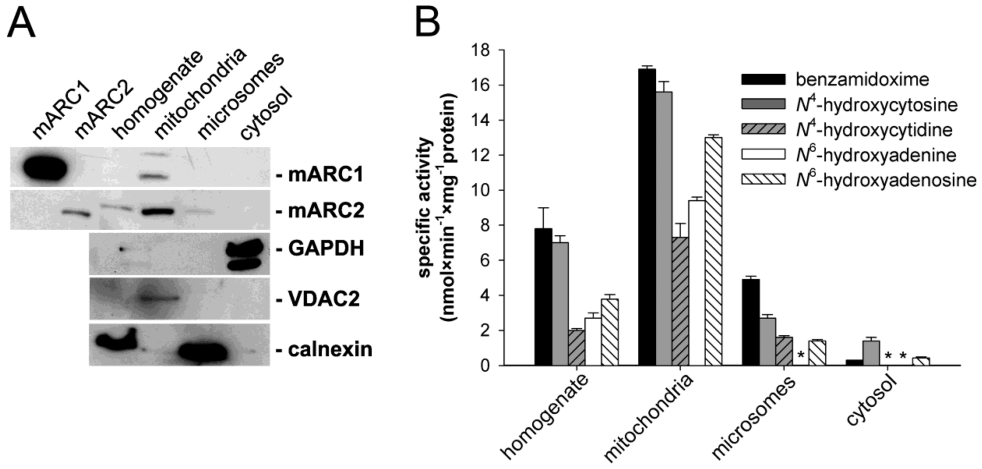


Figure 2. In vitro reduction of N-hydroxylated nucleobases and nucleosides by porcine subcellular fractions. (A) Western blot analysis of mARC in the subcellular hepatic fractions. Equal amounts of protein per lane were subjected to SDS-PAGE. For immunoblot analysis, anti-mARC1 antibody, anti-mARC2 antibody, anti-GAPDH antibody, anti-VDAC antibody, or anticalnexin antibody was used. Complete blots are presented in Figure S1 in the Supporting Information. (B) Complete incubation mixtures consisted of 0.05 mg of protein, 1 mM NADH, and 1.5−6 mM substrate in 150 μL of 100 mM phosphate buffer, pH 6.0. Conversion rates are means ± SDs of four determinants. Incubation, sample preparation, and HPLC analysis are described in detail in the Experimental Procedures (*, not detectable).

Table 1. In Vitro Reduction of N-Hydroxylated Nucleobases and Nucleosides by the Reconstituted N-Reductive Enzyme System[a]

		specific activity (nmol × min^{-1} × mg^{-1} total protein)				
mARC isoform	composition	N^4-hydroxycytosine[b]	N^4-hydroxycytidine	N^6-hydroxyadenine	N^6-hydroxyadenosine	benzamidoxime
recombinant mARC1	complete	118.9 ± 26.6	103.1 ± 38.7	180.8 ± 3.6	220.2 ± 26.0	159.4 ± 36.0
	without NADH	ND	ND	ND	ND	ND
	without cyt b_5	ND	ND	ND	ND	ND
	without cyt b_5R	ND	ND	ND	1.0 ± 0.1	4.4 ± 0.4
	without mARC1	ND	ND	ND	ND	ND
	limit of quantification	3.5	3.5	7.0	0.7	0.7
recombinant mARC2	complete	39.6 ± 2.7	50.2 ± 2.8	133.5 ± 2.0	105.3 ± 8.7	142.5 ± 24.4
	without NADH	ND	ND	ND	ND	ND
	without cyt b_5	ND	ND	ND	ND	ND
	without cyt b_5R	ND	ND	ND	0.9 ± 0.1	2.4 ± 0.2
	without mARC2	ND	ND	ND	ND	ND
	limit of quantification	3.7	3.7	7.3	0.7	0.7

[a]A complete incubation mixture consisted of 7.6 pmol of cyt b_5R, 76 pmol of cyt b_5, and 76 pmol of recombinant mARC1 or mARC2, 1 mM NADH, and 3−6 mM substrate in 150 μL of 20 mM MES buffer, pH 6.0. Conversion rates are means ± SDs of four determinations. Incubation, sample preparation, and HPLC analysis are described in detail in the Experimental Procedures (ND, not detectable). [b]Data previously published in Wahl et al.[9]

in the range of 10−500 μM of the metabolites in the presence of protein matrix and the cosubstrates NADH and NAD$^+$. The separation of all compounds was achieved within short run times (<11.5 min) and with good resolution characteristics (>3.2). Representative chromatograms for the separation of the N-hydroxylated analogues and their reduced metabolites are displayed in Figure 1, showing incubation mixtures with heat-denaturated protein spiked with the indicated N-hydroxylated base analogue and its respective metabolite.

Characterization of Proteins Used for in Vitro Incubation. To assess the purity of the subcellular fractions, Western blots using antibodies against microsomal (calnexin),[24] mitochondrial (VDAC),[25] and cytosolic (GAPDH)[26] marker proteins were carried out (Figure 2A). To characterize the N-reductive activity of the protein batches used, incubations with the well-established mARC marker substrate benzamidoxime were performed under identical conditions as for the base analogues (Figure 2B).

The cofactor saturation of recombinant proteins was analyzed by different spectroscopic techniques as described in the Experimental Procedures and showed 7% heme saturation for cyt b_5, 95% FAD saturation for cyt b_5R, and 28 or 32% molybdenum saturation for mARC1 or mARC2, respectively. In a reconstituted enzyme system with cyt b_5 and cyt b_5R, both mARC proteins were capable of reducing the model substrate benzamidoxime to benzamidine with similar conversion rates. The highest conversion rates were measured with the complete enzyme system, while omitting one of the components, including NADH, resulted in a nearly complete loss of activity (Table 1).

N-Reductive Activity Is Enriched in Mitochondria. The reduction of the N-hydroxylated pyrimidine and purine nucleobases as well as their nucleosides was studied in porcine subcellular liver fractions. Incubations with purified fractions led to the formation of the corresponding nucleobases or nucleosides, which were quantified by HPLC (Figure 1).

Table 2. Kinetic Parameters of the Reduction of N-Hydroxylated Nucleobases and Nucleosides[a]

substrate	mARC isoform	K_m (mM)	V_{max} (nmol × min^{-1} × mg^{-1})	catalytic efficiency (M^{-1} × s^{-1})
N^4-hydroxycytosine	mARC1	0.52 ± 0.14	174.1 ± 12.6	2080
	mARC2	1.52 ± 0.28	65.3 ± 4.4	256
	porcine liver mitochondria	0.92 ± 0.07	11.2 ± 0.3	–
N^4-hydroxycytidine	mARC1	0.44 ± 0.04	130.2 ± 3.2	1838
	mARC2	5.65 ± 0.51	111.1 ± 5.4	117
	porcine liver mitochondria	1.02 ± 0.07	8.2 ± 0.2	–
N^6-hydroxyadenine	mARC1	0.77* ± 0.20	375.1* ± 51.5	3026
	mARC2	0.72 ± 0.11	120.2 ± 5.7	996
	porcine liver mitochondria	0.16* ± 0.02	21.9* ± 0.6	–
N^6-hydroxyadenosine	mARC1	0.98 ± 0.11	279.8 ± 10.8	1774
	mARC2	5.28 ± 1.26	210.9 ± 17.1	522
	porcine liver mitochondria	0.63 ± 0.15	17.8 ± 1.1	–

[a]The incubation mixture consisted of 7.6 pmol of cyt b5R, 76 pmol of cyt b5, and 76 pmol of recombinant mARC1 or mARC2 in 150 μL of 20 mM MES buffer, pH 6.0, or 0.05 mg of porcine liver mitochondria in 100 mM phosphate buffer, pH 6.0, respectively, 1 mM NADH, and variable concentrations of substrate. Conversion rates are means ± SDs of four determinants. Incubation, sample preparation, and HPLC analysis are described in detail in the Experimental Procedures (*, calculated).

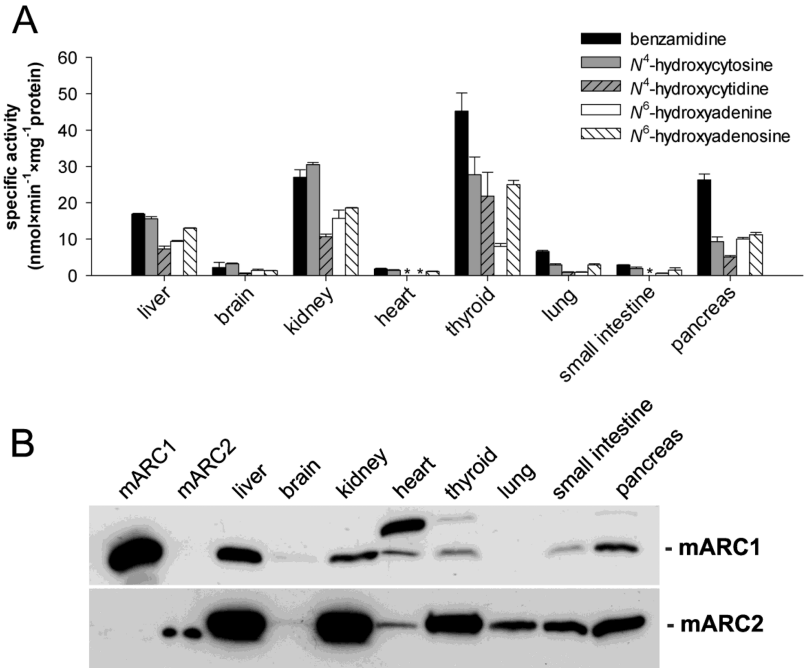


Figure 3. In vitro reduction of N-hydroxylated nucleobases and nucleosides by porcine hepatic and extrahepatic mitochondria. (A) Complete incubation mixtures consisted of 0.01−0.1 mg of protein, 1 mM NADH, and 1.5−6 mM in 150 μL of 100 mM phosphate buffer, pH 6.0. Conversion rates are means ± SDs of four determinants. Incubation, sample preparation, and HPLC analysis are described in detail in the Experimental Procedures (*, not detectable). (B) Western blot analysis of mARC in the porcine hepatic and extrahepatic mitochondria. Equal amounts of mitochondrial fraction per lane were subjected to SDS-PAGE. For immunoblot analysis, anti-mARC1 or anti-mARC2 antibody was used. Complete blots are presented in Figure S2 in the Supporting Information.

Moreover, all reactions showed strict dependency on NADH as cosubstrate. The in vitro reduction with subcellular fractions was most efficient in the mitochondrial fraction, whereas only minor reduction rates were found in the microsomal and cytosolic fractions. Because enrichment of the reductase activity for the N-hydroxylated base analogues in the mitochondrial fraction was consistent with the enrichment of mARC marker activity (BAO reduction) (Figure 2B), it is indicated that all used substrates were converted by the mARC-containing enzyme system. This is also confirmed by the enrichment of both mARC proteins in the mitochondrial fractions as demonstrated by use of specific antibodies (Figure 2A).

Highest Reduction Rates in Liver, Kidney, Thyroid, and Pancreas. As N-reductive activity was enriched in the mitochondrial fraction, V_{max} and K_m were determined by incubation of liver mitochondria at varying substrate concentrations of the tested base analogues. All reactions followed Michaelis−Menten kinetics with K_m values <1 mM of all tested substrates. The highest V_{max} values were observed for N^6-hydroxyadenine and its corresponding nucleoside (Table 2). However, using N^6-hydroxyadenine as the substrate, the N-reductive activity was inhibited at concentrations above 1 mM. Therefore, parameters of the reduction with N^6-hydroxyadenine were calculated using the linear plot of Linewaever−Burk in the range of 0.1−1.0 mM.

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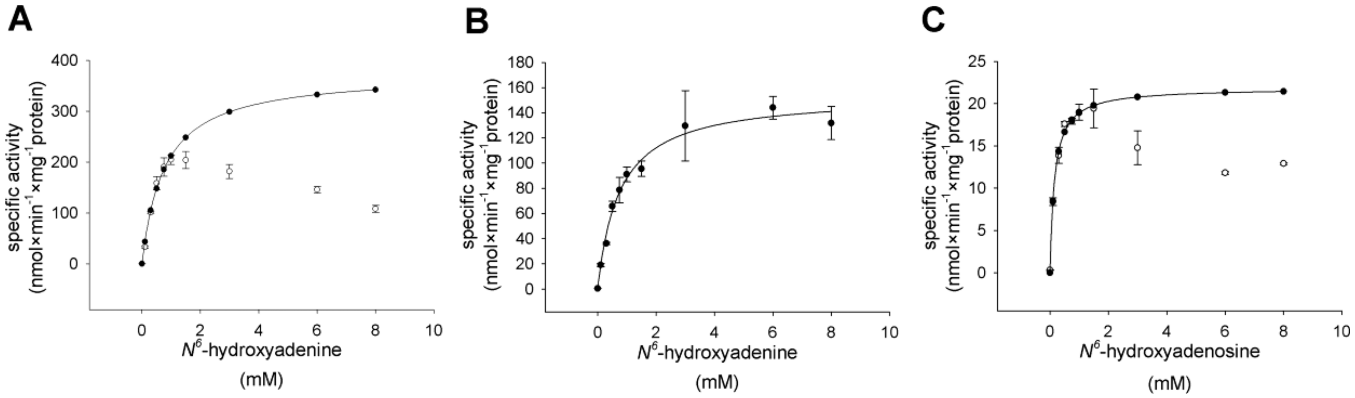

Figure 4. Michaelis–Menten curves of N^6-hydroxyadenine with mARC1 (A), mARC2 (B), and porcine liver mitochondria (C). The incubation mixture consisted of 7.6 pmol of cyt b_5R, 76 pmol of cyt b_5, and 76 pmol of recombinant mARC1 or mARC2 in 150 μL of 20 mM MES buffer, pH 6.0, or 0.05 mg of porcine liver mitochondria in 100 mM phosphate buffer, pH 6.0, respectively, 1 mM NADH, and variable concentrations of substrate. Conversion rates are means ± SDs of four determinants. Incubation, sample preparation, and HPLC analysis are described in detail in the Experimental Procedures.

Furthermore, the N-reductive activities of mitochondria from extrahepatic tissues were investigated and compared to those of the hepatic fractions. In fact, the N-reductive activity could be observed in all analyzed extrahepatic mitochondria. However, depending on the substrate, mitochondria from various tissues exhibited different conversion rates, with the highest reduction rates in mitochondrial fractions of thyroid, kidney, pancreas, and liver (Figure 3A). This is in agreement with the expression pattern of both mARC isoforms (Figure 3B).

Detoxification of N-Hydroxylated Nucleobases and Nucleosides Is Based on the mARC-Containing Enzyme System. The reduction of a N-hydroxylated purine nucleobase as well as N-hydroxylated purine and pyrimidine nucleosides was studied using recombinantly expressed enzyme sources. Incubations with the reconstituted enzyme system consisting of cyt b_5, cyt b_5R, and either mARC1 or mARC2 led to the formation of the corresponding nucleobases or nucleosides. As shown for the porcine liver fractions, the reaction was strictly dependent on NADH. Furthermore, the highest conversion rates were only detected with the complete enzyme system, whereas the lack of one of the components either resulted in strongly decreased or completely abrogated activity (Table 1).

All reactions followed Michaelis–Menten kinetics, which is shown for N^6-hydroxyadenine as an example (Figure 4). Although all tested N-hydroxylated nucleobases and nucleosides were reduced by mARC1 and mARC2, differences in the catalytic efficiency were observed. In general, V_{max} values were higher and K_m values were lower when mARC1 was used, indicating a higher affinity of mARC1 to the tested nucleobase analogues. For both proteins, the highest V_{max} values were observed for N^6-hydroxyadenine and its corresponding nucleoside (Table 2). As already observed with liver mitochondria (Figure 4C), substrate concentrations above 1 mM led to a reduction of the specific activity of mARC1 (Figure 4A) but not of mARC2 (Figure 4B).

DISCUSSION

In this study, for the first time, we provide evidence for the contribution of the mammalian molybdenum enzyme mARC to the detoxification of toxic and mutagenic N-hydroxylated nucleobases and nucleosides. All hitherto analyzed mammalian genomes harbor two mARC genes, mARC1 (MOSC1) and mARC2 (MOSC2), which are highly conserved on nucleic acid and amino acid levels.[9] It has been shown earlier that both human mARC proteins are able to reduce N-hydroxylated substrates such as benzamidoxime. Both mARC proteins are not active as stand-alone protein but require reconstitution with cyt b_5 and cyt b_5R (Table 1). The function of cyt b_5R is likely associated with the oxidation of NADH for recruitment of electrons, whereas cyt b_5 carries the electrons from cyt b_5R to mARC and mARC represents the active site where the respective N-hydroxylated substrate is reduced.[5,8,9] mARC is found in mitochondria,[5,9] which is confirmed in the present study by detecting and discriminating both mARC homologues in mitochondria from various porcine tissues using isoform-specific antibodies (Figure 3B). Thus, it is not surprising that the N-reductive activity for the marker substrate benzamidoxime is enriched in mitochondrial fractions in comparison to liver homogenates (Figure 2B). To identify a possible contributing role of the mitochondrial three-component enzyme system to the reduction of N-hydroxylated base analogues and the corresponding nucleosides, coincubation studies with porcine subcellular fractions and various N-hydroxylated base analogues were carried out.

In fact, enrichment of N-reductive activity in the mitochondrial fractions for N-hydroxylated base and nucleoside analogues was comparable to that of the (mARC-specific) marker substrate benzamidoxime, suggesting that the reduction of benzamidoxime and N-hydroxylated base analogues is catalyzed by identical proteins in a similar manner. In support of this, the expression pattern of both mARC isoforms in porcine mitochondria of distinct tissues correlates with the high reductase activities in thyroid, pancreas, kidney, and liver (Figure 3). We therefore conclude that the mARC-containing enzyme system is responsible not only for reduction of the marker substrate benzamidoxime but also for the tested N-hydroxylated base analogues. In fact, the involvement of the mARC-containing enzyme system in the detoxification reactions was confirmed using the complete recombinant human three-component system: after reconstitution with the electron transport proteins cyt b_5 and cyt b_5R, both recombinant mARC1 and mARC2 were able to reduce the hydroxylamines, whereas incubation mixtures lacking one of these proteins showed no or only negligible activity (Table 1). On the basis of the arrangement of redox centers and its similarity to eukaryotic nitrate reductases of plants, fungi, and

algae, it can be assumed that reduction of the tested N-hydroxylated substrates by the mARC-containing enzyme system occurs via cleavage of the N−O bond as proposed for nitrate reductase during conversion of nitrate to nitrite.[11]

Kinetic studies performed with the recombinant human enzymes revealed that all base and nucleoside analogues are very good substrates for the mARC-containing enzyme system. The highest conversion rates of N^6-hydroxyadenine and its nucleoside were determined with both the porcine liver mitochondria and the reconstituted human enzyme system, the latter presenting a specific reductase activity about 15 times higher than that of the mitochondrial fractions. Strikingly, the human mARC1-containing system showed higher maximum rates (V_{max}) and smaller Michaelis−Menten constants (K_m) than the enzyme system containing human mARC2 (Table 2). In addition, K_m values of mARC1 and hepatic mitochondria are similar (Table 2), which indicates that one of the two mARC proteins, presumably the mARC1 isoform, is of particular relevance to the reductive detoxification pathway in vivo. Moreover, there seems to be a slight substrate preference comparing the reduction of nucleosides derivatives and free bases, as reduction rates of nucleosides are faster when reduction is catalyzed by mARC1. However, in case mARC2 is used in the incubation mixture, higher conversion rates of free bases were determined. In this regard, another observation is noteworthy: incubation series with concentrations above 1 mM N^6-hydroxyadenine resulted in inhibition of the N-reductive activity in both the mitochondria and the recombinant human mARC1-containing enzyme system. In contrast, the reconstituted system with human mARC2 was not affected by high substrate concentrations of this purine derivative (Figure 4A−C). We speculate that inhibition by high substrate concentrations is a specific reaction of the mARC1 homologue, supporting a contribution of mARC proteins to detoxification of N-hydroxylated base analogues. However, further experiments are needed to determine whether both or only one mARC isoform is involved in this N-reductive pathway of N-hydroxylated DNA bases in vivo. Using a knock-down approach, Neve and co-workers recently postulated that only mARC2 is involved in the N-reductive pathway in murine cell culture.[10] However, RNAi studies in HEK-293 cells performed in our lab clearly indicate that knock-down of human mARC1 expression significantly diminishes reductase activity (unpublished results, manuscript in preparation).

In summary, we showed that N-hydroxylated base analogues were converted by the mammalian enzyme system, and both recombinant human mARC proteins are able to catalyze this reduction in vitro. Interestingly, reduction of N^6-hydroxyadenine has also been reported earlier for another mammalian molybdenum enzyme, that is, xanthine oxidoreductase (in the form of xanthine oxidase).[27] However, because in the present work the N-reductive activity was shown to be enriched in mitochondria, the mARC-dependent pathway appears more significant than the cytosolic pathway involving xanthine oxidoreductase.

In general, N-reduction seems to be a detoxification mechanism, which protects the cells against the mutagenic and toxic effects of N-hydroxylated base analogues such as those used in the present study. We propose that the N-reductive enzyme system protects the pathway of nucleobases from N-hydroxylated analogues and therefore from incorporation of modified nucleotides into DNA and RNA. In support of this assumption, similar detoxification pathways involving MOSC domain-carrying proteins were reported in other organisms such as the green algae *Chlamydomonas reinhardtii*[28] and *E. coli*.[29−31] As shown in the present work, two Moco-binding MOSC proteins have been found to participate in detoxification of N-hydroxylated base analogues in *E. coli*, with the YcbX protein together with its redox partner protein CysJ representing the more relevant system as compared to the YiiM protein with as yet unknown redox partner.[29−31] Although detoxification of N-hydroxylated base analogues is not necessarily the only function of eukaryotic and bacterial MOSC domain proteins, it is likely to represent one of the physiologically relevant functions.

■ ASSOCIATED CONTENT

Supporting Information

Western blot analysis of subcellular hepatic fractions and Western blot analysis of mARC1 and mARC2 in the porcine hepatic and extrahepatic mitochondria. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

Moco, molybdenum cofactor; MOSC, Moco sulfurase C-terminal domain; GAPDH, glycerinaldehyde-3-phosphate dehydrogenase; VDAC, voltage-dependent anion channel; IgG, immunoglobuline G; ECL, enhanced chemiluminescence; BCA, bicinchoninic acid; MES, 2-morpholinoethanesulfonic acid

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