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Functional Characterization of Protein Variants Encoded by Nonsynonymous Single Nucleotide Polymorphisms in *MARC1* and *MARC2* in Healthy Caucasians

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ABSTRACT

Human molybdenum-containing enzyme mitochondrial amidoxime reducing component (mARC), cytochrome b_5 type B, and NADH cytochrome b_5 reductase form an *N*-reductive enzyme system that is capable of reducing *N*-hydroxylated compounds. Genetic variations are known, but their functional relevance is unclear. Our study aimed to investigate the incidence of nonsynonymous single nucleotide polymorphisms (SNPs) in the mARC genes in healthy Caucasian volunteers, to determine saturation of the protein variants with molybdenum cofactor (Moco), and to characterize the kinetic behavior of the protein variants by in vitro biotransformation studies. Genotype frequencies of six SNPs in the mARC genes (c.493A>G, c.560T>A, c.736T>A, and c.739G>C in *MARC1*; c.730G>A and c.735T>G in *MARC2*) were determined by pyrosequencing in a cohort of 340 healthy Caucasians. Protein variants were expressed in *Escherichia coli*. Saturation with Moco was determined by measurement of molybdenum by inductively coupled mass spectrometry. Steady state assays were performed with benzamidoxime. The six variants were of low frequency in this Caucasian population. Only one homozygous variant (c.493A; *MARC1*) was detected. All protein variants were able to bind Moco. Steady state assays showed statistically significant decreases of catalytic efficiency values for the mARC-2 wild type compared with the mARC-1 wild type ($P < 0.05$) and for two mARC-2 variants compared with the mARC-2 wild type (G244S, $P < 0.05$; C245W, $P < 0.05$). After simultaneous substitution of more than two amino acids in the mARC-1 protein, *N*-reductive activity was decreased 5-fold. One homozygous variant of *MARC1* was detected in our sample. The encoded protein variant (A165T) showed no different kinetic parameters in the *N*-reduction of benzamidoxime.

Introduction

Mitochondrial amidoxime reducing component (mARC) was discovered in 2006, and is the fourth molybdenum-containing enzyme in humans (Havemeyer et al., 2006). There are two known isoforms of mARC: mARC-1 and mARC-2. These isoforms are encoded by two genes (*MARC1,* NM_022746.3; and *MARC2,* NM_017898.3), which are located on chromosome 1 (1q41) in a tandem arrangement. Their sequences show 66% identity and 80% similarity (Wahl et al., 2010).

The mARC proteins contain a mitochondrial targeting signal sequence, a predicted β-barrel domain, and a molybdenum cofactor sulfurase C-terminal domain- containing (MOSC) domain (Anantharaman and Aravind, 2002). The MOSC domain is characterized by a conserved cysteine in positions 273 and 272 of mARC-1 and mARC-2, respectively (Anantharaman and Aravind, 2002; Hille et al., 2011). mARC proteins contain molybdenum, which requires coordination by a pyranopterin to gain biologic activity. This complex is named molybdenum cofactor (Moco) and serves as a prosthetic group of mARC. Eukaryotic molybdenum enzymes are classified by the coordination chemistry of the molybdenum atom of Moco in two groups: the xanthine oxidoreductase family and the sulfite oxidase family. mARC proteins are regarded as members of the sulfite oxidase family (Chamizo-Ampudia et al., 2011; Havemeyer et al., 2011; Rajapakshe et al., 2011; Mendel and Kruse, 2012).

The subcellular localization of mARC proteins is not fully defined (Hille et al., 2011). Previous studies in tissues of different species showed that at least one isoform of mARC was detectable in mitochondria (Da Cruz et al., 2003; Havemeyer et al., 2006; Wahl et al., 2010; Havemeyer et al., 2011; Krompholz et al., 2012; Neve et al., 2012). mARC-2 is also localized in peroxisomes (Islinger et al., 2007; Wiese et al., 2007). Studies with human cell lines revealed that mARC-1 is associated with the outer mitochondrial membrane with an $N_{(in)}$-$C_{(out)}$ membrane orientation. The catalytic domain is localized at the C-terminus and exposed to the cytosol (Klein et al., 2012).

Molybdenum-containing enzymes in general show redox activity (Hille et al., 2011). mARC-1 and mARC-2 exert *N*-reductive activity together with cytochrome b_5 type B (*CYB5B*, NM_030579.2) and NADH cytochrome b_5 reductase (*CYB5R3*, NM_007326.4) in the

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ABBREVIATIONS: ANOVA, analysis of variance; CYB5B, cytochrome b_5 type B (outer mitochondrial membrane); CYB5R3, cytochrome b_5 reductase 3; HPLC, high-performance liquid chromatography; ICP-MS, inductively coupled mass spectrometry; mARC, mitochondrial amidoxime reducing component; Moco, molybdenum cofactor; SNP, single nucleotide polymorphism; WT, wild-type.

presence of NADH (Havemeyer et al., 2006). Binding of Moco is essential for the *N*-reductive activity of mARC. Wild-type (WT) proteins of mARC-1 and mARC-2 expressed in the *Escherichia coli* strains RK5204 and RK5206 do not contain Moco; consequently, *N*-reductive activity is absent after reconstitution in the in vitro assay together with cytochrome b_5 type B and NADH cytochrome b_5 reductase (Wahl et al., 2010).

The physiologic role of mARC and the *N*-reductive enzyme system is still not known (Havemeyer et al., 2011). The mARC-containing *N*-reductive enzyme system catalyzes the reduction of *N*-hydroxylated substrates (Havemeyer et al., 2011). A putatively physiologic substrate is the nitric oxide precursor N^{ω}-hydroxy-L-arginine, which is involved in the complex regulation of nitric oxide biosynthesis (Kotthaus et al., 2011). Other putative substrates are *N*-hydroxylated base analogs. Their reduction indicates a role of mARC in detoxification pathways (Wahl et al., 2010; Krompholz et al., 2012). Moreover, amidoximes (*N*-hydroxyamidines) can be used as prodrugs of amidines by lowering the basicity of the amidines and thus preventing a positive charge and low permeability. In vivo they are activated by reduction to amidines by the mARC-containing *N*-reductive enzyme (Clement, 2002). Drugs carrying this prodrug principle include ximelagatran as well as several drug candidates in development (Clement and Lopian, 2003; Peterlin-Masic et al., 2006; Froriep et al., 2013). Benzamidoxime is used as a model compound for this prodrug principle (Clement, 2002).

Single nucleotide polymorphisms (SNPs) are known in both mARC genes. At the beginning of our studies, six nonsynonymous SNPs in *MARC1* and two in *MARC2,* localized in the open reading frame for the *N*-truncated soluble recombinant proteins, were registered in the National Center for Biotechnology Information Single Nucleotide Polymorphism database (dbSNP, release build 133; http://www.ncbi.nlm.nih.gov/snp/). There are currently 41 nonsynonymous SNPs in *MARC1* and 27 in *MARC2* (dbSNP build 138). To the best of our knowledge, no studies have been published to date demonstrating whether the resulting amino acid changes have any functional consequences for the variant protein.

This study aimed to investigate the prevalence of nonsynonymous SNPs in both mARC genes in healthy Caucasians, to determine saturation with Moco of recombinant expressed human mARC proteins (WT and variants) by measurement of molybdenum content, and to characterize the enzyme kinetics of genetic mARC variants by in vitro biotransformation studies using the model substrate benzamidoxime (Fig. 1).

Materials and Methods

Subjects. Genotype and allele frequencies of four SNPs in *MARC1* and two SNPs in *MARC2* were determined in DNA samples from a cohort of 340 nonrelated, healthy Caucasian volunteers of German origin (110 men and 230 women; mean age 33.3 years; range, 26–67 years). All subjects gave their written informed consent and this study was approved by the Ethics Committee of the Medical Faculty of Christian-Albrechts-University of Kiel.

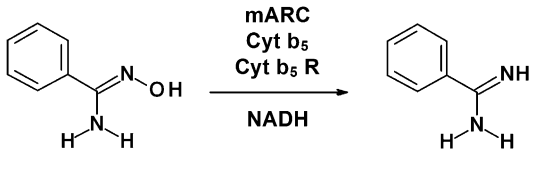

Fig. 1. Reduction of benzamidoxime by the mARC-containing *N*-reductive enzyme system. Benzamidoxime is reduced to benzamidine by mARC, cytochrome b_5 type B (Cyt b_5), and NADH cytochrome b_5 reductase (Cyt b_5 R) in the presence of NADH.

DNA Isolation. Whole genomic DNA was isolated from venous blood samples by using the QIAamp DNA Blood Mini Kit (Qiagen, Hildesheim, Germany).

Genotyping. National Center for Biotechnology Information dbSNP release builds 130–133 were reviewed for SNPs in human *MARC1* and *MARC2*. SNPs were selected for genotyping according to the following criteria: nonsynonymous SNPs, localization in the open reading frame for the *N*-truncated soluble recombinant proteins, and notified SNPs not present in Caucasians only in conjunction with a SNP, which fulfilled the above-mentioned criteria.

The following SNPs were determined by pyrosequencing on a PSQ 96HS platform (Biotage AB, Uppsala, Sweden): c.493A>G (T165A, rs2642438), c.560T>A (M187K, rs17850677), c.736T>A (C246S, rs3738178), and c.739G>C (D247H, rs72470601) for *MARC1*; and c.730G>A (G244S, rs3795535) and c.735T>G (C245W, rs76664695) for *MARC2*.

Primers were designed by Pyrosequencing Assay Design software (version 1.0; Biotage AB), and primer sequences are listed in Table 1. PCR was carried out in a thermocycler in a reaction volume of 25 μl containing 2.5 μl 10 × PCR Rxn buffer - $MgCl_2$ (Invitrogen, Darmstadt, Germany), 1.25 μl $MgCl_2$ (50 mM; Invitrogen), 2.5 μl dNTP (2.5 mM; Biozym, Hessisch Oldendorf, Germany), 0.5 μl primer F (10 μM; Sigma-Aldrich, Steinheim, Germany), 0.5 ml primer R (10 μM; Sigma-Aldrich), 0.15 μl Taq DNA Polymerase (Invitrogen), 1 μl genomic DNA, and ultra-pure water (Invitrogen). Thermocycler (Gene Amp PCR System 9700, version 3.08; Applied Biosystems, Darmstadt, Germany) conditions consisted of initial denaturation for 2 minutes at 95°C followed by 50 cycles of 30 seconds at 95°C, 30 seconds at 65°C (c.493A>G, c.560T>A, c.730G>A, c.735T>G) and 63°C (c.736T>A, c.739G>C), respectively, and 30 seconds at 72°C, terminal elongation for 7 minutes at 72°C, and a final hold at 14°C. Agarose gel electrophoresis (7 μl PCR product, 2% agarose gel, 120 V, 30 minutes) was performed to check the length of the DNA fragment against a DNA ladder (GeneRuler 100bp DNA Ladder; Fermentas, Darmstadt, Germany). Pyrosequencing followed a standard protocol as essentially published previously (Haenisch et al., 2008).

Construction of Expression Vectors. Expression vectors were constructed as previously described (Wahl et al., 2010). Variants c.286G>C, c.493G>A, c.560T>A, c.736T>A, c.739G>C, and c.804G>A of *MARC1* and c.730G>A and c.735T>G of *MARC2* were generated by PCR mutagenesis using primers carrying the desired mutation. Variants leading to multiple amino acid changes in mARC-1 were also generated by PCR mutagenesis. The six amino acid changes were introduced in six steps using primers carrying the desired mutation. The order of the mutations was c.493G>A followed by c.560T>A, c.286G>C, c.736T>A, c.739G>C, and c.804G>A in *MARC1*. Accuracy of PCR mutagenesis was confirmed by DNA sequencing (GATC, Konstanz, Germany).

Expression and Purification of Recombinant Proteins. Standard expression of the mARC proteins and variants was performed in freshly transformed *E. coli* TP 1000 cells. Cells were supplemented with 1 mM sodium molybdate. Expression of cytochrome b_5 type B was performed in *E. coli* DL41. Cells were supplemented with 1 mM aminolevulinic acid to support heme synthesis. Expression of NADH cytochrome b_5 reductase was performed in *E. coli* DL41. A detailed description of expression and purification was previously published (Wahl et al., 2010; Kotthaus et al., 2011).

Determination of Protein Concentrations. Concentrations of all recombinant proteins were determined by a BCA assay after precipitating proteins using the Compat-Able Protein Assay Preparation Reagent Set (Thermo Scientific, Waltham, MA) and Pierce BCA Protein Assay Kits (Thermo Scientific). All samples were measured at 562 nm (Cary 50; Varian Inc., Belrose, Australia). Measurement of each duplicate was repeated twice.

Determination of Cofactor Contents of the Recombinant Human Proteins. Molybdenum content of the mARC proteins was measured by inductively coupled plasma mass spectrometry (ICP-MS) as described earlier (Kotthaus et al., 2011). The analytical error as determined from replicates and certified reference materials was better than 3% RSD (relative standard deviation; 1σ). mARC proteins were eluted in elution buffer (50 mM sodium phosphate, pH 8.0, containing 300 mM sodium chloride, 250 mM imidazole, and 10% glycerol). Molybdenum content of mARC proteins was determined after exchanging the buffer of the mARC proteins to exclude contamination with sodium molybdate that was added for expression of the proteins. Initially, molybdenum content of the proteins was measured with and without exchanging the buffer to 50 mM potassium phosphate, pH 7.4, containing

TABLE 1
Primers used for genotyping of four SNPs in MARC1 and two SNPs in MARC2

SNP	Primer Sequence	*T*m	Fragment Length of PCR Product
		°C	*bp*
MARC1			
c.493A>G	F: 5′-Bio-AGTGCACGGCCTGGAGATAGAG	74.0	84
	R: 5′-TAGGGCTGTGACTTCAGGAAGCT	72.6	
	S: 5′-CTGGTTATCCACTGG	51.7	
c.560T>A	F: 5′-Bio-GCCCAGTGGATAACCAGCTTC	72.0	109
	R: 5′-TGGGTCGGAACAAGTCTGCTATT	73.0	
	S: 5′-GGACGTCTCGGTCGC	59.2	
c.736T>A	F: 5′-Bio-CTCAACTCCAGGCTAGAGAAGAA	68.1	107
c.739G>C	R: 5′-GATCCAAAGGGGCATAGTGTTAC	69.7	
	S: 5′-TGTTACCTCTGCATAGACA	51.7	
MARC2			
c.730G>A	F: 5′-AGATGCCTCCCTGGTAGATTTGAA	73.1	108
	R: 5′-Bio-CTTACCTCCTCAAAAGCATCACAG	70.3	
	S: 5′-GCCAAATATTGTGGTGAC	53.9	
c.730G>A	F: 5′-Bio-TGCCTCCCTGGTAGATTTGAATA	70.8	120
c.735T>G	R: 5′-AAAAGCAGTGGGTCGCTTACCTC	73.7	
	S: 5′-TACCTCCTCAAAAGCAT	50.4	

Bio, biotin; F, forward primer used for PCR; R, reverse primer used for PCR; S, sequencing primer used for pyrosequencing; *T*m, melting temperature.

20% (m/v) glycerol, 0.1 mM dithiothreitol, and 1 mM sodium EDTA (Roth, Karlsruhe, Germany). After ensuring that there was no difference between these two methods of sample preparation, proteins were measured without exchanging the buffer. Measurement of each duplicate was repeated three times. Molybdenum saturation was calculated from molybdenum content and calculated molecular masses of the mARC proteins.

Cytochrome b_5 type B contains an N-terminal heme binding domain carrying 1 mol heme/mol protein (Parthasarathy et al., 2011). Heme content was determined by measurement of difference spectra according to Estabrook and Werringloer (1978). Cytochrome b_5 type B was reduced in the presence of NADH cytochrome b_5 reductase by NADH (Merck, Darmstadt, Germany). A wavelength scan from 400 to 500 nm was run (Cary 50). Absorption showed a maximum at 426 nm and a minimum at 409 nm. The addition of NADH was repeated until the difference between maximum and minimum of absorption reached a maximum. Heme content was calculated from the maximal difference between maximum and minimum absorption using the molar extinction coefficient for the absorbance change at 426 nm − 409 nm (ε = 185 $mM^{-1} \times cm^{-1}$). The assay was performed in duplicate. Heme content of the lots of cytochrome b_5 type B used in this work ranged from 2.1 to 2.9 nmol heme/mg protein.

NADH cytochrome b_5 reductase contains a flavoprotein domain carrying 1 mol FAD/mol protein (Kensil and Strittmatter, 1986). FAD content was determined according to Whitby (1953). Samples were denatured at 100°C for 10 minutes in the dark. Proteins were sedimented by centrifugation (10 minutes, 10,000 rpm). Then, 200 μl supernatant of each sample was transferred to a 96-well plate. Absorption was measured at 450 nm (Cary 50). Calculation of FAD content was carried out using calibration in the range of 0.01–0.1 μmol FAD/ml with an accuracy of the mean of 99.1% ± 3.1%. The assay was performed in duplicate. FAD content of NADH cytochrome b_5 reductase used in this work was 16 nmol FAD/mg protein.

Benzamidoxime. Benzamidoxime (*N*-hydroxy-benzene-carboximidamide) was synthesized from benzonitrile and hydroxylamine as previously described and was assayed as usual (Krüger, 1885).

Determination of Kinetic Parameters of the mARC Variants. Incubation mixtures were adjusted on the cofactors of the three recombinant proteins. High values of *N*-reductive activity were measured when high amounts of mARC and cytochrome b_5 type B and low amounts of NADH cytochrome b_5 reductase were added to incubation mixtures. mARC containing 120 pmol molybdenum, cytochrome b_5 type B containing 60 pmol heme, and NADH cytochrome b_5 reductase containing 6 pmol FAD were incubated in steady state assays with 0.1–5 mM benzamidoxime in 20 mM 4-morpholineethanesulfonic acid, pH 6.0. Incubations were performed under aerobic conditions at 37°C. After 3 minutes of preincubation, the reaction was started by adding 1 mM NADH and was stopped after 15 minutes by adding ice-cold methanol. Precipitated proteins were separated by centrifugation (5 minutes, 10,000 rpm) and the amount of product in the supernatant was determined by high-performance liquid chromatography (HPLC). Reduction followed Michaelis–Menten kinetics. Data were initially checked for substrate inhibition by using Lineweaver–Burk plots. Kinetic parameters V_{max} and K_m were determined through Michaelis–Menten plots using SigmaPlot 11.0 (Systat Software, Chicago, IL). In cases of substrate inhibition, kinetic parameters were calculated from data of the linear range of the Lineweaver–Burk plots. Each lot of mARC protein was incubated three times in duplicate and measured twice by HPLC. Incubations of multiple variants of mARC contained duplicates that were measured twice.

Separation of Benzamidoxime from Benzamidine by HPLC. An HPLC system was built comprising a Waters 1525 HPLC pump, a Waters 717 autosampler, and a Waters 2487 dual absorbance detector combined with Waters Breeze software version 3.30 (Waters Corporation, Milford, MA). HPLC analysis was performed on a LiChroCART 250-4 LiChrospher60 RP-select B (5 μm) column and a LiChroCART 4-4 guard column (Merck, Darmstadt, Germany). Elution was carried out isocratically with 10 mM 1-octylsulfonate sodium salt (Tokyo Chemical Industry, Tokyo, Japan) and 17% (v/v) acetonitrile (Sigma-Aldrich). pH was not adjusted. The flow rate was kept at 1 ml/min. Characteristic retention times were 7.9 ± 0.1 minutes for benzamidoxime and 26.7 ± 0.1 minutes for benzamidine (Clement et al., 2005).

Statistical Analysis. Data analysis of statistically significant differences was performed with SigmaPlot 11.0 using a *t* test or one-way analysis of variance (ANOVA) followed by a suitable post hoc test. $P < 0.05$ was considered significant.

Results

Genotype Frequencies of SNPs in *MARC1* and *MARC2*. The SNPs c.493A>G, c.560T>A c.736T>A, and c.739G>C in *MARC1* and c.730G>A and c.735T>G in *MARC2* were determined by pyrosequencing. It was possible to detect variant genotypes in 292 to 320 samples of the investigated cohort of 340 individuals. Frequency distribution of alleles and genotypes is shown in Table 2.

For *MARC1*, the frequencies of all genotypes were in Hardy–Weinberg equilibrium. The most frequently detected SNP was c.493A>G; however, variant genotypes 493AG and GG were observed more frequently than the reference genotype 493AA, which had a frequency of only 7.1%. Heterozygous variants of c.560T>A and c.736T>A were detected in 2.7% and 0.3% of the samples,

TABLE 2

Allele and genotype frequencies of nonsynonymous SNPs in *MARC1* and *MARC2*

SNP (Amino Acid Change)	Allele	Frequency[a]	dbSNP Frequency[b]	Genotype	Frequency[a]	dbSNP Frequency[b]
		%	%		%	%
MARC1						
c.493A>G	A	30.4	28.3	A/A	7.1	10.0
rs2642438	G	69.6	71.7	A/G	46.6	36.7
(T165A)				G/G	46.2	53.3
c.560T>A	T	98.6	No data	T/T	97.3	No data
rs17850677	A	0.4	No data	T/A	2.7	No data
(M187K)			No data	A/A	0.0	No data
c.736T>A	T	99.8	100.0	T/T	99.7	100.0
rs3738178	A	0.2	0.0	T/A	0.3	0.0
(C246S)				A/A	0.0	0.0
c.739G>C	G	100.0	97.6	G/G	100.0	95.2
rs72470601	C	0.0	2.4	G/C	0.0	4.8
(D247H)				C/C	0.0	0.0
MARC2						
c.730G>A	G	99.5	99.6	G/G	99.1	99.1
rs3795535	A	0.5	0.4	G/A	0.9	0.9
(G244S)				A/A	0.0	0.0
c.735T>G	T	100.0	86.1	T/T	100.0	No data
rs76664695	G	0.0	13.9	T/G	0.0	No data
(C245W)				G/G	0.0	No data

[a]Frequency data determined in a cohort of 340 healthy Caucasians.
[b]Frequency data published in dbSNP build 133. Frequency data of rs2642438, rs17850677, rs3738178, rs72470601, and rs3795535 were provided by HapMap.

respectively, whereas no homozygous carriers were identified. c.739G>C was not detected in the cohort.

For *MARC2*, only heterozygous variants for c.730G>A were detected in 0.9% of the samples. c.735T>G was not detected in the cohort.

No further variants were found in carriers of the homozygous *MARC1* 493AA genotype. Two samples of the cohort carried two heterozygous variants. One sample was heterozygous for c.493A>G and c.560T>A in *MARC1*. Another sample was heterozygous for c.493A>G in *MARC1* and c.730G>A in *MARC2*.

Molybdenum Content of the Recombinant Human mARC Proteins. All protein variants of mARC-1 and mARC-2 were successfully expressed in *E. coli* and the WT and variants had similar expression levels.

All mARC variants were able to bind Moco. Molybdenum content of WT and variant mARC protein with one amino acid exchange could be determined in the range of 14–29 nmol molybdenum per milligram protein (Fig. 2). The mARC-1 WT and variants showed a molybdenum content of 22 ± 4 nmol molybdenum/mg protein, representing a molybdenum saturation of 71% ± 14%. The mARC-2 WT and variants had a molybdenum content of 20 ± 4 nmol molybdenum/mg protein, representing a molybdenum saturation of 72% ± 12%. There was no statistically significant difference in molybdenum content between WT and variant protein (ANOVA followed by Dunnett's post hoc test; SigmaPlot 11.0).

Molybdenum content of mARC-1 WT and variants with more than one amino acid exchange could be determined in the above-mentioned range for mARC-1. Values of these protein lots lay between 18 and 26 nmol molybdenum/mg protein (Fig. 3). The mean value of molybdenum content was 22 ± 2 nmol molybdenum/mg protein, representing a molybdenum saturation of 74% ± 8%.

Kinetic Parameters of mARC WT and Variants. Reduction of benzamidoxime was carried out with recombinant mARC WT and variants, cytochrome b_5 type B, and NADH cytochrome b_5 reductase and increasing amounts of the substrate in the presence of NADH. The reduction followed Michaelis–Menten kinetics. Arithmetic means of V_{max} and K_m and their standard deviations are shown in Table 3.

V_{max} of mARC-2 variant C245W was significantly decreased compared with WT ($P < 0.050$, ANOVA followed by Dunnett's post hoc test; SigmaPlot 11.0).

Catalytic efficiency was calculated as k_{cat}/K_m from kinetic parameters. Arithmetic means of the values and their standard deviations are shown in Fig. 4.

Catalytic efficiency of mARC-1 WT was 1370 ± 430 s^{-1} M^{-1}. Catalytic efficiency of mARC-2 WT (890 ± 150 s^{-1} M^{-1}) was statistically significant different compared with mARC-1 WT (P = 0.005, t test; SigmaPlot 11.0). Both variants of mARC-2, G244S (580 ± 40 s^{-1} M^{-1}) and C245W (650 ± 180 s^{-1} M^{-1}), showed a statistically significant decrease of catalytic efficiency compared

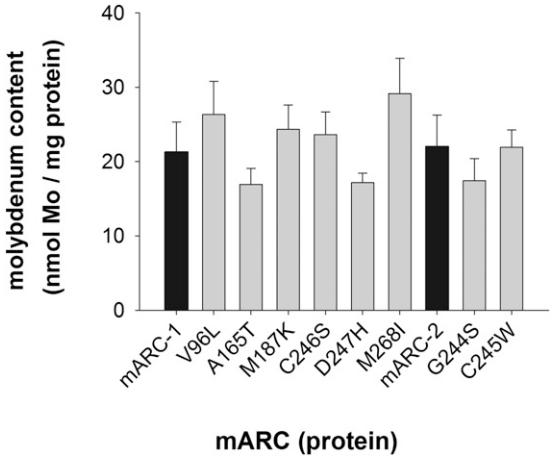

Fig. 2. Molybdenum content of mARC WT and variants. Molybdenum content was measured by ICP-MS as described in *Materials and Methods*. Data are means of quadruplicates ± S.D. The black bars present WT proteins, whereas the gray bars present variant proteins. Mo, molybdenum.

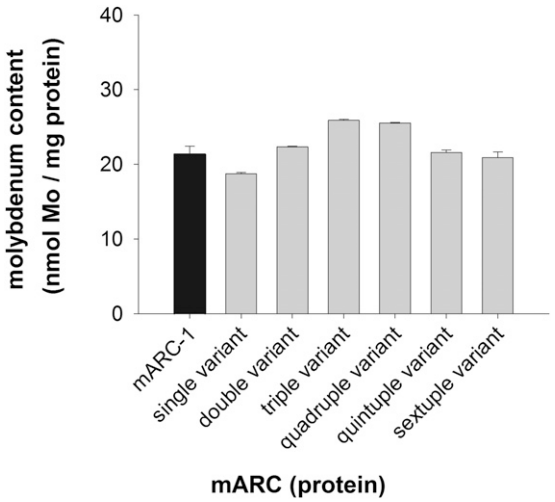

Fig. 3. Molybdenum content of mARC-1 WT and multiple variants. Amino acid substitutions A165T, M187K, V96L, C246S, D247H, and M268I were introduced successively in mARC-1 WT. Molybdenum content was measured by ICP-MS as described in *Materials and Methods*. Data are means of duplicates ± S.D. The black bar presents the WT protein, whereas the gray bars present variant proteins.

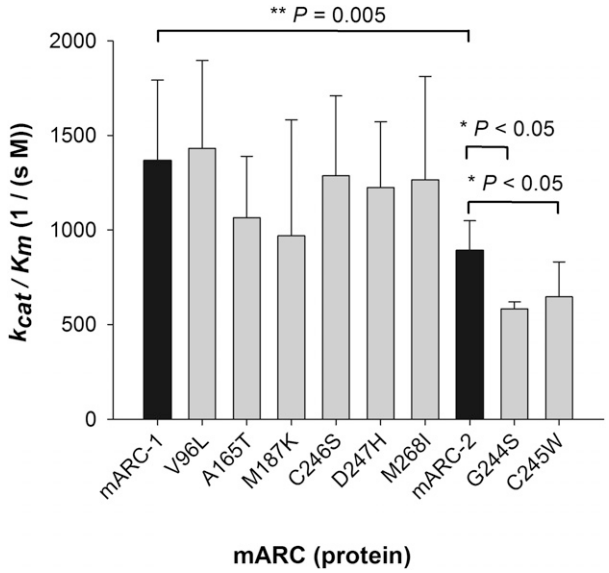

Fig. 4. Catalytic efficiency of mARC WT and variants. mARC containing 120 pmol molybdenum, cytochrome b_5 type B containing 60 pmol heme, and NADH cytochrome b_5 reductase containing 6 pmol FAD were incubated with 0.1–5 mM benzamidoxime in 20 mM 4-morpholineethanesulfonic acid, pH 6.0, for 15 minutes under aerobic conditions. Triplicates of each mARC protein were incubated in duplicate and measured twice by HPLC. Catalytic efficiency was calculated from kinetic parameters as described in *Materials and Methods*. Data are means ± S.D. of the number of protein lots mentioned in Table 3. The black bars present WT proteins, whereas the gray bars present variant proteins.

with mARC-2 WT ($P < 0.050$, ANOVA followed by Dunnett's post hoc test; SigmaPlot 11.0).

***N*-Reductive Activity of Multiple Variants of mARC-1.** Kinetic parameters were determined for mARC-1 WT and variants, in which amino acid substitutions A165T, M187K, V96L, C246S, D247H, and M268I were successively introduced. V_{max} decreased almost constantly from WT to the sextuple variant, in which all six amino acids were exchanged. K_m increased almost constantly in this order (Table 4). Catalytic efficiency also decreased in this order of variants and was almost absent in the sextuple variant (Fig. 5). Simultaneous incubation of the WT and all variants confirmed this continuous decrease in specific *N*-reductive activity (data not shown).

Discussion

Genotyping of genetic variants in both mARC genes revealed that most of them had a low frequency in this Caucasian population. An exception was *MARC1* c.493A>G (rs2642438), which had an allele frequency of 71.7% for the variant allele; hence, this variant should actually be considered as the WT rather than variant. These data are consistent with frequency data of several other studies in Caucasian populations published in the dbSNP. The 735G allele was not detected in our cohort, although dbSNP build 133 reported an allele frequency of 13.9%. However, later dbSNP builds removed this information.

Carriers of more than one variant were rare. In carriers of the homozygous variant *MARC1* 493AA, no other variants were detected. Only two subjects carrying two heterozygous variants were observed in the cohort of 340 healthy Caucasians. One subject was heterozygous for c.493A>G and c.560T>A in *MARC1*. Another was heterozygous for c.493A>G in *MARC1* and c.730G>A in *MARC2*.

The latest release of the SNP database (dbSNP build 138) published 41 nonsynonymous missense SNPs in *MARC1* and 27 in *MARC2*. Most of the data were provided from huge sequencing projects. Frequency data are published for 25 of the 41 nonsynonymous SNPs in *MARC1*, but 17 SNPs are absent among Caucasians. Of the published allele frequencies in *MARC2* in Caucasians, frequency data are published for only three nonsynonymous SNPs in *MARC2*.

Based on the information on the presence of genetic variants in *MARC1* and *MARC2* in Caucasians, we were interested in whether the proteins had different binding properties of Moco and different catalytic properties.

All components of the *N*-reductive enzyme system were expressed as truncated soluble recombinant proteins in *E. coli*. The predicted N-terminal mitochondrial targeting sequences of the mARC proteins, the membrane-anchoring domain of cytochrome b_5 type B, and the membrane-binding domain of NADH cytochrome b_5 reductase were removed to improve expression (Wahl et al., 2010). Using *E. coli* TP1000 as an expression system of mARC proteins ensures that the eukaryotic form of Moco is expressed (Palmer et al., 1996). The simple incubation of all three proteins is a useful tool for in vitro metabolism studies and offers the possibility of analyzing the catalytic ability of recombinant expressed mARC variants until a

TABLE 3

V_{max} and K_m values of the reduction of benzamidoxime by mARC WT and variants

Data are the means ± S.D. of three different incubations of each protein lot.

mARC Protein	Protein Lots	V_{max} [a]	K_m [a]
	n	*nmol BA/min per mg protein*	*mM BAO*
mARC-1			
mARC-1	4	105 ± 23	0.2 ± 0.1
V96L	1	138 ± 24	0.3 ± 0.1
A165T	2	125 ± 28	0.4 ± 0.2
M187K	2	104 ± 22	0.3 ± 0.1
C246S	2	93 ± 21	0.2 ± 0.1
D247H	2	113 ± 18	0.3 ± 0.1
M268I	1	94 ± 18	0.2 ± 0.1
mARC-2			
mARC-2	3	119 ± 34	0.4 ± 0.1
G244S	1	84 ± 10	0.4 ± 0.1
C245W	2	48 ± 11 [b]	0.2 ± 0.1

BA, benzamidine; BAO, benzamidoxime.

[a] Each mARC protein was incubated in duplicate and measured twice by HPLC. Kinetic parameters were determined as described in *Materials and Methods*.

[b] Statistical significant decrease of V_{max} compared with WT ($P < 0.050$, ANOVA followed by Dunnett's post hoc test; Sigma Plot 11.0)

TABLE 4

V_{max} and K_m values of the reduction of benzamidoxime by mARC-1 WT and multiple variants

Data are the means ± S.D.

mARC Protein[a]	Protein Lots	V_{max} [b]	K_m [b]
	n	*nmol BA/min per mg protein*	*mM BAO*
mARC-1	2	106 ± 25	0.2 ± 0.0
Single variant	1	82 ± 1	0.2 ± 0.0
Double variant	1	110 ± 1	0.3 ± 0.0
Triple variant	1	62 ± 2	0.5 ± 0.0
Quadruple variant	1	52 ± 1	0.4 ± 0.0
Quintuple variant	1	41 ± 5	0.4 ± 0.2
Sextuple variant	1	1 ± 0	0.7 ± 0.2

BA, benzamidine; BAO, benzamidoxime.

[a]Amino acid substitutions A165T, M187K, V96L, C246S, D247H, and M268I were introduced successively in mARC-1 WT.

[b]Each mARC protein was incubated in duplicate and measured twice by HPLC. Kinetic parameters were determined as described in *Materials and Methods*.

three-dimensional structure is available that shows how the enzyme system is anchored in the outer mitochondrial membrane (Havemeyer et al., 2011).

Similar in vitro metabolism studies were performed successfully with another molybdenum enzyme, human sulfite oxidase. Expression of recombinant human sulfite oxidase, in which its mitochondrial targeting signal was also removed, was optimized in *E. coli* TP 1000, leading to a high amount of active protein. Proteins with or without the His-tag did not show any differences in kinetic parameters (Temple et al., 2000). The R160Q variant causes sulfite oxidase deficiency in vivo. Expression of this variant by site-directed mutagenesis revealed a protein that was able to bind the prosthetic groups, Moco and heme, but showed a 1000-fold decrease of catalytic efficiency with the substrate sulfite in vitro (Garrett et al., 1998).

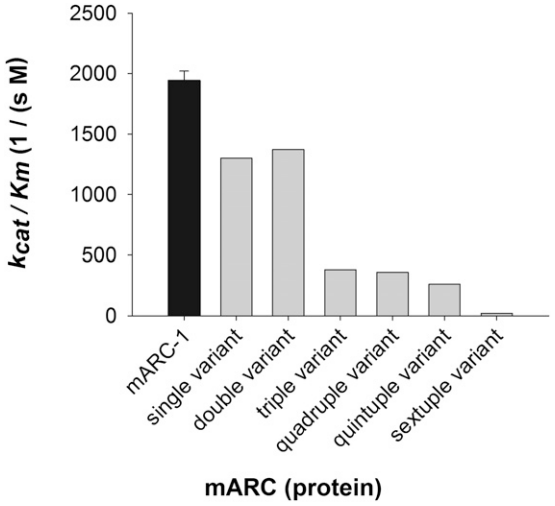

Fig. 5. Catalytic efficiency of mARC-1 WT and multiple variants. Amino acid substitutions A165T, M187K, V96L, C246S, D247H, and M268I were introduced successively in mARC-1 WT. mARC containing 120 pmol molybdenum, cytochrome b_5 type B containing 60 pmol heme, and NADH cytochrome b_5 reductase containing 6 pmol FAD were incubated with 0.1–5 mM benzamidoxime in 20 mM 4-morpholineethanesulfonic acid, pH 6.0, for 15 minutes under aerobic conditions. Each mARC protein was incubated in duplicate and measured twice by HPLC. Catalytic efficiency was calculated from kinetic parameters as described in *Materials and Methods*. Data are means ± S.D. of two lots of WT and means of one lot of each variant. The black bar presents the WT protein, whereas the gray bars present variant proteins.

Moco is the essential basis for *N*-reductive activity of mARC proteins (Wahl et al., 2010). For example, 1 mol Moco is bound to 1 mol mARC protein as a prosthetic group (Havemeyer et al., 2011; Hille et al., 2011). Each Moco contains an oxidized molybdenum atom in its center and is a highly unstable structure after liberation from the protein (Schwarz et al., 2009). Molybdenum content of the mARC proteins can easily be measured by ICP-MS. Results showed that the mARC WT and variants possess molybdenum, which can only be derived from Moco because molybdenum was absent in all negative controls. An mARC WT expressed in the *E. coli* strain RK5204 and a NADH cytochrome b_5 reductase purified after expression in a sodium molybdate-containing medium were used as negative controls. WT and protein variants of mARC-1 and mARC-2 were successfully expressed in *E. coli*, but differences in molybdenum content between the WT and variants were not detectable. Therefore, we conclude that the investigated nonsynonymous SNPs did not influence the binding of Moco to the variant protein.

Kinetic parameters of all mARC variants were determined. Steady state assays revealed that mARC-1 and mARC-2 follow Michaelis–Menten kinetics.

Statistically significant decreases of kinetic parameters were only detected in variants of mARC-2. The V_{max} of the C245W variant and catalytic efficiency values of the G244S and C245W variant ($P < 0.050$, ANOVA followed by Dunnett's post hoc test; SigmaPlot 11.0) were lower than in the WT. However, subjects carrying any homozygous variant genotype leading to the protein variants G244S and C245W were not detected in our sample.

K_m values of human mARC-2 were slightly higher than that of human mARC-1. This led to a statistically significant increase of catalytic efficiency of mARC-1 WT compared with mARC-2 WT ($P = 0.005$, *t* test; SigmaPlot11.0). Human mARC-1 seems to be the isoform of higher affinity and catalytic efficiency toward benzamidoxime. This is consistent with results of previous studies (Wahl et al., 2010; Krompholz et al., 2012). Catalytic efficiency is not influenced by variants of mARC-1. There were no statistically significant differences in kinetic parameters between mARC-1 WT and mARC-1 variants. In addition, the A165T variant of mARC-1, which was detected in our sample, did not show any different kinetic parameters.

Because a single SNP in *MARC1* was not able to induce a loss of function in the mARC-1 protein, multiple variants were recombinantly expressed to investigate whether they exert greater influence on the *N*-reductive activity. Indeed, analysis of multiple variants of mARC-1 showed an almost constant decrease in the catalytic efficiency from the WT to sextuple variants (Fig. 5).

After replacements of more than two amino acids in mARC-1, catalytic efficiencies were at least 5-fold decreased compared with the WT. This result suggests that more than two amino acids have to be changed in the variant mARC protein to induce differences in *N*-reductive activity.

In sextuple variants, *N*-reductive activity was almost absent although Moco was bound to the protein. Possibly all of the investigated amino acid changes did not influence the binding of Moco but together might participate in binding of the substrate benzamidoxime.

The double variant of mARC-2, which contained the amino acid changes G244S and C245W, was expressed in *E. coli* and was found to bind Moco. Catalytic efficiency was not further decreased as observed for the C245W variant (data not shown).

The mARC enzyme system is important for drug development and detoxification. On the one hand, the *N*-reductive enzyme system is able to activate prodrugs with an amidoxime structure (Clement, 2002). Oral bioavailability is important for the drug compliance of patients and helps reduce costs in health care systems compared with

parenterally applied drugs. Therefore, safe prodrug strategies without any interindividual variability in activation are needed. On the other hand, the *N*-reductive enzyme system is able to reduce toxic and mutagenic *N*-hydroxylated nucleobases and their corresponding nucleosides and might be involved in protection of cellular DNA from misincorporation of toxic *N*-hydroxylated base analogs (Krompholz et al., 2012).

The oral direct thrombin inhibitor ximelagatran was an approved drug that contained an amidoxime structure. Initially, metabolism was investigated and three metabolites of ximelagatran were detected (Eriksson et al., 2003). During clinical studies of long-term treatment, liver enzyme elevations occurred. No evidence was found for additional metabolism pathways or the formation of reactive metabolites during the reduction of ximelagatran to melagatran, which might lead to an increase of plasma levels of alanine aminotransferase (Kenne et al., 2008). A genetic association between elevated alanine aminotransferase levels and the major histocompatibility complex allele DRB1*0701 was discovered, which suggests a possible immune mechanism of this adverse drug reaction and not any connection to the prodrug principle and the reduction of ximelagatran to melagatran (Kindmark et al., 2008).

The mARC-containing enzyme system only needs an *N*-hydroxy structure for the reduction. Several studies provide evidence that *N*-oxygenated structures are reduced independent from their structure (e.g., benzamidoxime, *N*-hydoxymelagatran, *N*-hydroxy-sulfonamides, *N*-hydroxy-valdecoxib, *N*-hydroxycytosine, *N*-hydroxycytidine, *N*-hydroxyadenine, *N*-hydroxyadenosine, and upamostat) (Gruenewald et al., 2008; Havemeyer et al., 2010; Wahl et al., 2010; Krompholz et al., 2012; Froriep et al., 2013) and are catalyzed by the same mARC-containing enzyme system. Consistent with these data and based on the assumption that truncation might not affect the protein structure and activity, our results can offer the simple conclusion that an adverse drug reaction due to frequent polymorphisms at the *MARC* loci and the possible reduced or inability to activate prodrugs might be unlikely.

This investigation characterized the influence of mARC variants on *N*-reductive activity. A previous study showed that the function of cytochrome b5 type B in the *N*-reductive pathway was not affected by the variants 2SF, D14G, K16E, and T22A (Plitzko et al., 2013). The functional relevance of variants of NADH cytochrome b_5 reductase has to be ruled out.

Further effects caused by the anchoring of the protein in the outer mitochondrial membrane or by posttranslational modifications of the proteins cannot be excluded. Although we could prove a significant effect of nonsynonymous SNPs on catalytic properties, the clinical significance remains unclear due to the rareness of variants in the general population.

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Authorship Contributions

Participated in research design: Ott, Reichmann, Boerger, Cascorbi, Bittner, Mendel, Kunze, Clement, Havemeyer.

Contributed new reagents or analytic tools: Cascorbi.

Performed data analysis: Ott, Boerger.

Wrote or contributed to the writing of the manuscript: Ott, Cascorbi, Bittner, Clement, Havemeyer.

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