The Fourth Molybdenum Containing Enzyme mARC: Cloning and Involvement in the Activation of *N*-Hydroxylated Prodrugs

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The recently discovered mammalian molybdoprotein mARC1 is capable of reducing *N*-hydroxylated compounds. Upon reconstitution with cytochrome b_5 and b_5 reductase, benzamidoxime, pentamidine, and diminazene amidoximes, *N*-hydroxymelagatran, guanoxabenz, and *N*-hydroxydebrisoquine are efficiently reduced. These substances are amidoxime/*N*-hydroxyguanidine prodrugs, leading to improved bioavailability compared to the active amidines/guanidines. Thus, the recombinant enzyme allows prediction about in vivo reduction of *N*-hydroxylated prodrugs. Furthermore, the prodrug principle is not dependent on cytochrome P450 enzymes.

Introduction

Basic functional groups such as amidines and guanidines are incorporated into various drug candidates and drugs. Because of their basicity, these structures are protonated under physiological conditions and are not absorbed from the gastrointestinal tract. *N*-hydroxylation of amidines leads to compounds which are less basic and not protonated under physiological conditions. Because these properties result in sufficient oral absorption and therefore in improved bioavailability the prodrug concept amidoximes instead of amidines was developed.[1] This prodrug principle was applied to peptidomimetic inhibitors and anticoagulants like melagatran[2,3] and nearly 50 compounds in preclinical and clinical development.[4] Furthermore, amidines are structural elements of trypanocidal and leishmanicidal compounds such as pentamidine and diminazene.[5,6] The guanidine structure is also well represented in numerous drug classes because its cation built by the protonation is often responsible for the interaction with the negatively charged carboxylic function of target molecules. The amidine and guanidine groups imitate the structural part of arginine. The prodrug principle developed for amidines can be applied to the guanidine structures as well.[7]

This study should terminate a long debate on the enzymatic basis of the reduction of these *N*-hydroxylated compounds. In older studies, Kadlubar and Ziegler[8,9] described a microsomal and Bernheim[10−12] a mitochondrial system consisting of cytochrome b_5 and NADH cytochrome b_5 reductase and a third unidentified component. The microsomal system was further analyzed and a cytochrome P450[a] enzyme was identified in pig liver microsomes as the third component.[13] So far the orthologous human P450 isoenzyme has not been found. For the mitochondrial enzyme system, the third component has further been analyzed until it was purified from pig liver mitochondria.[14]

For 40 years, it was believed that only three molybdenum containing enzymes are present in the human body. With the recently purified protein from pig liver mitochondria named as mitochondrial amidoxime reducing component, it became clear that a fourth molybdenum containing enzyme exists which might play a major role in drug metabolism though its physiological relevance is not still clear.[14]

This mARC protein is unique as it forms the catalytic part of a three-component enzyme complex. The *N*-reductive complex located in the outer mitochondrial membrane with its mARC protein represents the first eukaryotic molybdenum enzyme with separate electron transport proteins. As the human genome codes for two mARC-proteins, mARC1 and mARC2, differences and similarities in function and substrate specificity should be determined. In this study, the cloning, expression and purification of mARC1 is described. Furthermore, the *N*-reductive ability of the recombinant protein is demonstrated by the reduction of different amidine and guanidine prodrugs.

Results

Expression and Purification of Recombinant mARC1. Expression of mARC1 in *Escherichia coli* yielded a His_6-tagged protein with a molecular mass of approximately 40 kDa (Figure 1A), which corresponded well to the calculated mass of 38.5 kDa. As in previous works, the activity of mARC was shown to depend on molybdenum cofactor (Moco),[14] the *E. coli* strain TP1000 was chosen for recombinant expression of mARC1. *E. coli* TP1000 is characterized by high accumulation of the eukaryotic form of Moco, whereby sufficient saturation of recombinant mARC1 with Moco in the required form should be ensured. In fact, FormA-dephospho, the stable oxidation product of both Moco and its metal-free precursor molybopterin (MPT), could be identified on mARC1 (Figure 1B), indicating that recombinant human mARC1 was purified in the cofactor-loaded form. mARC1 was further analyzed for biologically active Moco by use of the *nit-1* reconstitution assay. This assay is based on the transfer of Moco derived from an exogenous source to the nitrate reductase apoprotein of the Moco-deficient *Neurospora crassa nit-1* mutant, whereby reconstitution of NADPH-dependent nitrate reductase activity is achieved.[15] When omitting supplementary molybdate from the reaction

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[a] Abbreviations: BAO, benzamidoxime; P450, cytochrome P450; cyt, cytochrome; DAO, diamidoxime; MAO, monoamidoxime; mARC, mitochondrial amidoxime reducing component; Moco, molybdenum cofactor; MPT, molybdopterin; OMV, outer membrane vesicles; b_5, cytochrome b_5; b_5 reductase, NADH cytochrome b_5 reductase.

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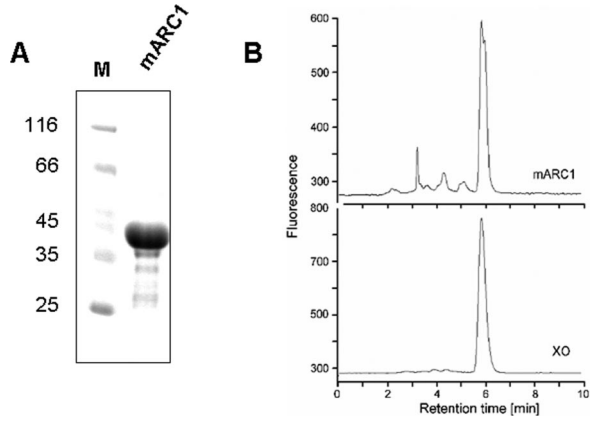

Figure 1. SDS-PAGE analysis of recombinant mARC1 and detection of Moco/MPT bound to mARC1. (A) Recombinant human mARC1 (40 μg) purified by affinity chromatography was electrophoresed on a 10 % SDS-PA gel and stained with Coomassie Brilliant Blue (M = molecular weight marker; masses are indicated in kDa). (B) Detection of Moco/MPT bound to recombinant human mARC1. Protein-bound Moco/MPT was converted into FormA-dephospho and detected via HPLC by its specific fluorescence (excitation, 370 nm; emission, 450 nm). The dominant peak eluting at ~6 min co-elutes with FormA-dephospho prepared from the Mo-enzyme xanthine oxidase (XO), which served as control.

mixture, only active Moco but not Mo-free MPT can be detected as only Moco is capable of reconstituting NADPH-nitrate reductase activity. In the presence of molybdate however, MPT is nonenzymatically converted to Moco, thereby enabling reconstitution of NADPH-nitrate reductase activity as well. mARC1 as purified after heterologous expression in *E. coli* TP1000 yielded a specific NADPH-nitrate reductase activity of 2.7 ± 0.2 μmol nitrite/(mg·min) in the absence of molybdate, while in the presence of molybdate, a specific activity of 3.2 ± 0.3 μmol nitrite/(mg·min) was observed. These results indicate that more than 80 % of the total cofactor bound to mARC1 is represented by *nit-1*-active Moco, which was able to reconstitute NADPH-nitrate reductase activity in the *nit-1* extract.

mARC1 is Capable of Reducing Benzamidoxime. Incubation with 0.5 mM benzamidoxime with the reconstituted enzyme system containing NADH led to the formation of benzamidine. The formation of benzamidine was determined by comparison of the metabolite retention time with that of the synthetic material using HPLC analysis. The relative rate of in vitro reduction of benzamidoxime to benzamidine is shown in Table 1. The reaction was further specified by omitting single components. Minor reduction rates could be found if no cytochrome b_5 or NADH cytochrome b_5 reductase was added to mARC1 (Table 1). The incubation mixture with only cytochrome b_5 and NADH cytochrome b_5 reductase led to benzamidine in a moderate extent. By omitting both electron transport proteins, complete loss of mARC′s *N*-reductive activity was observed. No *N*-reductive activity was detectable without adding NADH (Table 1). The greatest conversion rates could be detected if all three components were combined in presence of NADH.

Reduction of *N*-Hydroxylated Compounds. The in vitro reduction by mARC1 of all tested *N*-hydroxylated compounds can be demonstrated (Table 2). Comparing different substrates, there are significant variations concerning the *N*-reductive activity of mARC1. All amidoxime prodrugs are reduced to their amidines. Benzamidoxime as the model substrate for the *N*-reductive activity is converted to benzamidine as also shown in Table 1. The reduction of diamidoxime prodrugs to monoamidoximes and their further reduction to amidines are demonstrated (Table 2). Pentamidine monoamidoxime reduction to pentamidine is catalyzed by mARC1 by an even higher reduction rate than pentamidine diamidoxime to the corresponding monoamidoxime and pentamidine. *N*-hydroxymelagatran is reduced to melagatran (Table 2). The highest reduction rates are achieved by in vitro assays with *N*-hydroxylated guanidines (guanoxabenz reduction to guanabenz; *N*-hydroxydebrisoquine to debrisoquine).

Table 1. *N*-Reduction of Benzamidoxime by the Reconstituted System[a]

composition of incubation mixture	activity nmol·min^{-1}·mg^{-1} mARC1	activity pmol·min^{-1}
complete system	1.30 ± 0.43[b]	130.0 ± 42.8
complete system without BAO	ND[c]	
without NADH	ND	
without cyt b_5	0.23 ± 0.00	22.9 ± 0.5
without b_5 reductase	0.20 ± 0.00	20.0 ± 0.0
without mARC1		22.0 ± 0.3
only mARC1	ND	

[a] A complete incubation mixture consisted of 200 pmol purified microsomal cytochrome b_5, 0.05 units of purified microsomal NADH cytochrome b_5 reductase (pig liver), 100 μg recombinant mARC1, 0.5 mM benzamidoxime and 1 mM NADH in 150 μL of 100 mM phosphate buffer, pH 6.0. For incubation, sample preparation and HPLC analysis see Experimental Section or Supporting Information. Data are means ± SD of four determinations. [b] Significant versus composition without cytochrome b_5 and without NADH cytochrome b_5 reductase with $p < 0.05$ according to Student′s *t*-test. [c] ND = not detectable.

Table 2. *N*-Reduction of *N*-Hydroxylated Compounds by the Reconstituted System[a]

substrate	metabolite	specific activity nmol·min^{-1}·mg^{-1} mARC1
benzamidoxime	benzamidine	1.30 ± 0.43
pentamidine MAO	pentamidine	6.59 ± 0.35
pentamidine DAO	pentamidine MAO	0.56 ± 0.04
pentamidine DAO	pentamidine	0.49 ± 0.12
diminazene DAO	diminazene MAO	4.31 ± 1.00
diminazene DAO	diminazene	0.05 ± 0.02
N-hydroxymelagatran	melagatran	0.48 ± 0.01
guanoxabenz	guanabenz	20.42 ± 4.56
N-hydroxydebrisoquine	debrisoquine	15.94 ± 1.10

[a] A complete incubation mixture consisted of 200 pmol purified microsomal cytochrome b_5, 0.05 units of purified microsomal NADH cytochrome b_5 reductase (pig liver), 100 μg mARC1, 0.5 mM substrate and 1 mM NADH in 150 μl 100 mM phosphate buffer, pH 6.0. For incubation, sample preparation and HPLC analysis see Experimental Section or Supporting Information. Data are means ± SD of four determinations.

Discussion

Many studies have been performed to identify the enzymes responsible for the reduction of *N*-hydroxylated structures.[9,13,16] The participation of cytochrome b_5 and NADH cytochrome b_5 reductase in these reductions has become clear in these studies.[16] Whether a third component would be able to increase the conversion rates or not has been questioned,[17−19] but the participation of a third component has been reported by independent groups.[8,10,13,16] While a P450 enzyme has repeatedly been postulated as the *N*-reductive component in pig liver microsomes,[13,20] the current study has shown that a molybdenum-containing enzyme is responsible for the mitochondrial enzymatic activity.[14] Additionally, the mitochondrial enzyme system has turned out to be far more efficient than the microsomal one.[13,14] Therefore, it can be supposed that the reduction of *N*-hydroxylated compounds in the human body is mainly

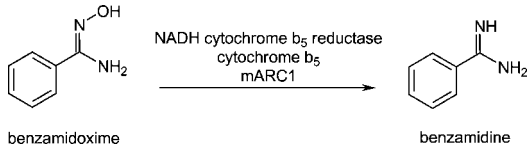

Figure 2. Reduction of the model substrate benzamidoxime to benzamidine by cytochrome b_5, NADH cytochrome b_5 reductase, and mARC1.

performed by mitochondria as well. The specific reduction rates of the recombinant mARC enzyme are comparable with the human liver mitochondria.[21] However, the third component has so far only been purified from pig liver mitochondria. The studies described in this work are for the first time based on the human mitochondrial enzyme, which has been obtained as a recombinant enzyme. The successfully demonstrated reductions show clearly that there is an enzyme system consisting of three components that reduces *N*-hydroxylated compounds in the human body. At this point, we identified the enzyme system responsible for mitochondrial *N*-reductive activity.

The in vitro system introduced in this study will be of great benefit for further characterizations of the metabolism of many *N*-hydroxylated compounds and for the development of novel *N*-hydroxylated prodrugs, especially of amidines and guanidines. Thus, the activation in the human body can be predicted in vitro. The importance of conversions by molybdenum containing enzymes will increase in the future because the conversion of novel drugs by P450 enzymes will be tried to be avoided because of potential interactions.[22]

The conversion rates indicate clearly that the highest conversion rates are obtained in the presence of all three components. This becomes especially obvious when the specific conversion rates are referred to constant amounts of cytochrome b_5 and NADH cytochrome b_5 reductase, respectively. The three component system also shows the highest conversion rates if the whole amount of protein in the incubation mixture is included. The ideal ratio of cytochrome b_5 and NADH cytochrome b_5 reductase has been claimed to be 10:1.[17] This ratio has been used for the studies described. The conversion could be increased by the addition of mARC to an incubation using the

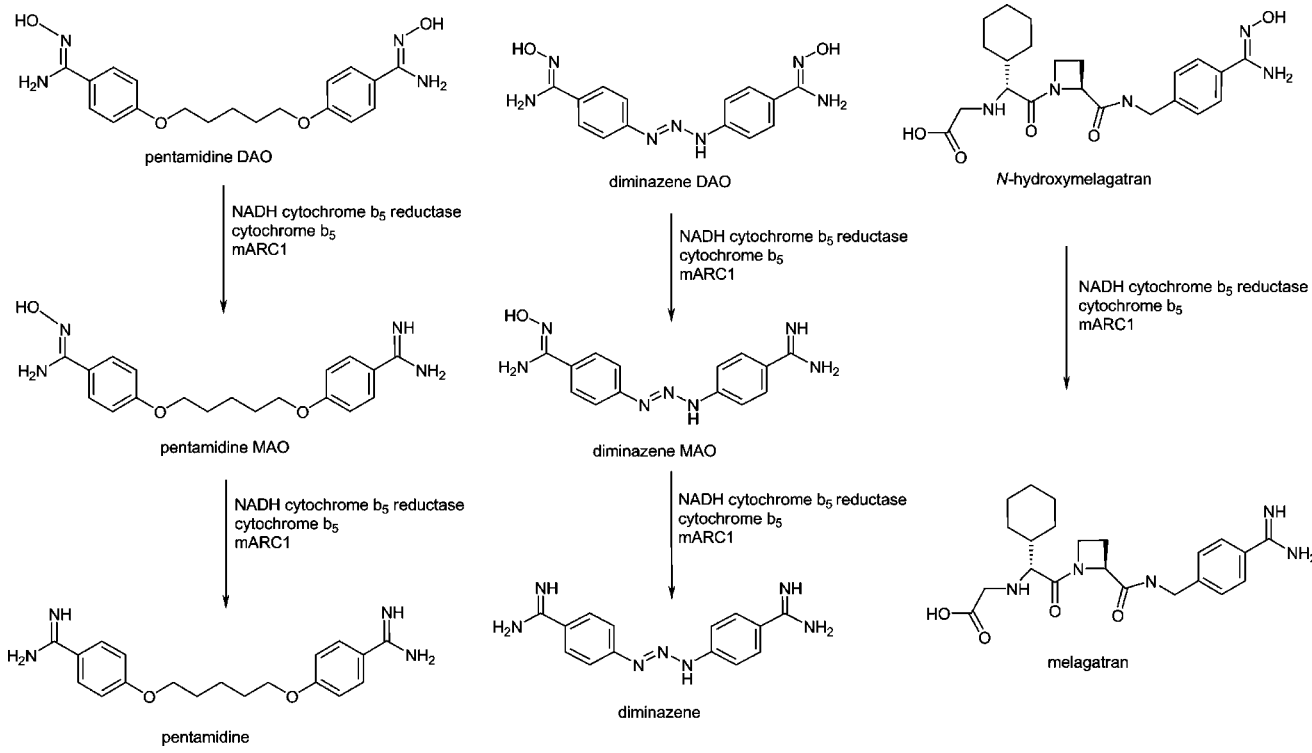

Figure 3. Reduction of amidoxime prodrugs to amidines by cytochrome b_5, NADH cytochrome b_5 reductase, and mARC1.

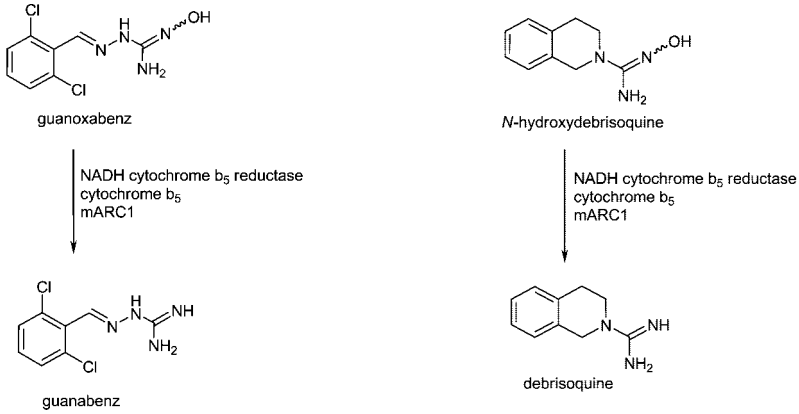

Figure 4. Reduction of *N*-hydroxylated guanidines to guanidines by cytochrome b_5, NADH cytochrome b_5 reductase, and mARC1.

proposed optimal ratio of cytochrome b_5 and NADH cytochrome b_5 reductase. Although microsomal and not mitochondrial cytochrome b_5 and NADH cytochrome b_5 reductase have been used for the studies described in this paper, it is very unlikely that this will influence the results. Outer mitochondrial membrane and microsomal cytochrome b_5 are products of different genes but demonstrate a large degree of homology.[23] In the case of NADH cytochrome b_5 reductase, the proteins are identical and arise from the same gene.[24]

Finally, the results shown present a fourth human molybdenum containing enzyme system in humans, which seems to be an excellent catalyst for reducing *N*-hydroxylated compounds. Whether this is also the physiological function has to be further investigated.

The details show that mARC is significantly involved in the activation of amidoxime prodrugs. The in vivo reduction of such amidoxime prodrugs has been proven by several clinical studies, especially with ximelagatran[2] and sibrafiban.[25] The simple benzamidoxime (Figure 2) and *N*-hydroxymelagatran (Figure 3) have been used as model substances in this study. It can be assumed that all other amidoxime prodrugs will be reduced in a similar manner. There are about 50 amidoxime prodrugs, which will ensure oral bioavailability of amidines in preclinical and clinical development, respectively.[4]

The prodrugs of pentamidine and diminazene (Figure 3) were used in this study as examples for orally available antitrypanosomal and antileishmanial bisamidines.[1] Besides derivatives of pentamidine and diminazene developed in our laboratories, other similar compounds like furamidine prodrugs[26] are in advanced clinical development. These amidoximes are methylated at the oxygen. Those methoxy compounds are initially dealkylated in order to expose the amidoxime. It can be assumed that the in vivo reduction of *N*-hydroxylated derivatives of furamidine is performed by the same three-component enzyme system and not only by cytochrome b_5 and NADH cytochrome b_5 reductase, as postulated.[19]

N-hydroxyguanidines, which are suited as prodrugs for guanidines,[7] are also converted by this enzyme system. In the present study, guanoxabenz and *N*-hydroxydebrisoquine (Figure 4) have been used as model substances and the highly efficient reduction has been shown. In this context, it can be speculated that the physiologically occurring *N*-hydroxy-L-arginine[27] is also reduced by this enzyme system. This is the purpose of presently ongoing research and might give further insight into a possible physiological role of mARC.

In the present study, for the first time, an enzyme system with a human recombinant third component has successfully been reconstituted and its contribution to the activation of amidoxime prodrugs has been demonstrated. It can serve as a model system in order to predict the in vivo situation and also to explain known reductions observed in vivo. The study also demonstrates that the prodrug principle amidoximes instead of amidines is not dependent on P450 enzymes. Thus, P450-dependent interactions are avoided, which also explains why those have not been observed for example in the case of ximelagatran.[2,3]

Amidines and guanidines are very often elements of new drug candidates as the cations formed after protonation interact with carboxylates of the target protein. However, these functional groups have negative pharmacokinetic properties. Replacements of amidines and guanidines result usually in a high loss of affinity. The prodrug principle amidoximes instead of amidines allows the maintenance of the amidine function and also oral bioavailability. This paper demonstrates that the activation of the amidoxime prodrugs is done by human enzymes not related to drug interactions.

Experimental Section

Materials. Benzamidoxime was synthesized from benzonitrile and hydroxylamine as described.[28] Pentamidine was obtained from Sanofi Aventis (Frankfurt, Germany). The pentamidine diamidoxime and monoamidoxime were synthesised in our laboratory as described by Clement and Raether[5] and Clement et al.[29] Melagatran and *N*-hydroxymelagatran were kindly supplied by AstraZeneca (Mölndal, Sweden). Diminazene and its amidoxime prodrugs were synthesized as described.[30] Debrisoquine sulfate was obtained from Sigma (Taufkirchen, Germany). *N*-Hydroxydebrisoquine was synthesized as described by Bailey and DeGrazia using dioxane instead of *N*,*N*-dimethylformamide in the last reaction step.[31] Guanabenz acetate was kindly supplied by Wyeth-Pharma GmbH (Muenster, Germany), guanoxabenz-HCl by Laboratoires Houdé (Paris, France). Acetonitrile, methanol, and acetic acid were purchased from J. T. Baker (Deventer, Holland). All other chemicals were commercially available and of analytical grade. Solvents used for HPLC were all of HPLC grade purity.

Cloning of Human mARC1. Total RNA prepared from human HepG2 cells by using the E.Z.N.A. Total RNA kit (Peqlab, Erlangen, Germany) was reverse transcribed with AMV-reverse transcriptase (Promega, Mannheim, Germany) and oligo-d(T) primer according to standard procedures. A *mARC1* cDNA was obtained by subsequent polymerase chain reaction using *mARC1*-specific primers mARC1_forward (5′-ATA TAT GGA TCC ATG GGC GCC GCC GGC TCC TCC GCG-3′) and mARC1-reverse (5′-AAA TTT AAG CTT TTA CTG GCC CAG CAG GTA CAC AGG-3′) deduced from GenBank entry NM_022746. By this procedure, a full-length open reading frame of 1011 base pairs was obtained encoding for a protein of 337 amino acids. In addition, restriction sites for *Bam*HI and *Hind*III were introduced at the 5′- and the 3′-ends, respectively, which enabled cloning of the *mARC1* cDNA into the pQE80 expression plasmid (Qiagen, Hilden, Germany) downstream from a sequence encoding six NH_2-terminal histidine residues. Correctness of the introduced cDNA was confirmed by sequencing. We note that a reproducible polymorphism was identified that resulted in substutition of lysine187 by methionine. However, this polymorphism is also found in the databases, e.g., between protein accessions AAH10619 and EAW93291, and thus, obviously resembles a naturally occuring polymorphism.

Expression and Purification of Recombinant mARC1. Routine protein expression of mARC1 was performed in freshly transformed *E. coli* TP1000 cells.[32] Cells were grown aerobically in Luria Broth medium in the presence of 100 μg/mL ampicillin at 22 °C to a $A_{600} = 0.1$ before induction with 15−30 μM isopropyl-β-D-thiogalactopyranoside and addition of 1 mM sodium molybdate. After induction, cells were grown for further 20 h at 22 °C. Cells were harvested by centrifugation and stored at −70 °C until use. Cell lysis was achieved by several passages through a French pressure cell, followed by sonication for 5 min on ice. After centrifugation, 6 × histidin-tagged protein was purified on a nickel−nitrilotriacetic acid superflow matrix (Qiagen, Hilden, Germany) under native conditions at 4 °C according to the manufacturer's manual. Eluted fractions were analyzed by SDS-PAGE. Molybdenum binding pterin bound to the purified proteins was detected and quantified by converting it to the stable oxidation product FormA-dephospho, according to Johnson and Rajagopalan.[33] Oxidation, dephosphory-lation, QAE chromatography, and high performance liquid chromatography (HPLC) analysis were performed as described previously.[34] The identity of Moco/MPT prepared as FormA-dephospho from mARC1 was proven by comparing its retention time with that of FormA-dephospho prepared from bovine xanthine oxidase (Sigma, Taufkirchen, Germany).

Assay for Determination of the Reduction Rate. Incubations were carried out under aerobic conditions at 37°C in a shaking water bath. If not otherwise stated, standard incubation mixtures of the reconstituted system contained 100 μg mARC1, 0.05 U NADH

cytochrome b_5 reductase, 200 pmol cytochrome b_5, 0.5 mM of the *N*-hydroxylated substrate, and 1.0 mM NADH in a total volume of 150 μL of 100 mM potassium phosphate buffer pH 6.0. After preincubation for 3 min at 37°C, the reaction was started by NADH and terminated after 15 or 20 min by adding aliquots of methanol. The precipitated proteins were sedimented by centrifugation and the supernatant was analyzed by HPLC.

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Supporting Information Available: Purification of cytochrome b_5 and NADH cytochrome b_5 reductase, enzyme assays, instrumentation (HPLC data for all compounds), and references. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Clement, B. Reduction of *N*-hydroxylated compounds: amidoximes (*N*-hydroxyamidines) as pro-drugs of amidines. *Drug Metab. Rev.* **2002**, *34*, 565–579.
(2) Gustafsson, D.; Nystrom, J.; Carlsson, S.; Bredberg, U.; Eriksson, U.; Gyzander, E.; Elg, M.; Antonsson, T.; Hoffmann, K.; Ungell, A.; Sorensen, H.; Nagard, S.; Abrahamsson, A.; Bylund, R. The direct thrombin inhibitor melagatran and its oral prodrug H 376/95: intestinal absorption properties, biochemical and pharmacodynamic effects. *Thromb. Res.* **2001**, *101*, 171–181.
(3) Clement, B.; Lopian, K. Characterization of in vitro biotransformation of new, orally active, direct thrombin inhibitor ximelagatran, an amidoxime and ester prodrug. *Drug Metab. Dispos.* **2003**, *31*, 645–651.
(4) Peterlin-Masic, L.; Cesar, J.; Zega, A. Metabolism-directed optimisation of antithrombotics: the prodrug principle. *Curr. Pharm. Des.* **2006**, *12*, 73–91.
(5) Clement, B.; Raether, W. Amidoximes of pentamidine: synthesis, trypanocidal and leishmanicidal activity. *Arzneimittelforschung* **1985**, *35*, 1009–1014.
(6) Bray, P. G.; Barrett, M. P.; Ward, S. A.; de Koning, H. P. Pentamidine uptake and resistance in pathogenic protozoa: past, present and future. *Trends Parasitol.* **2003**, *19*, 232–239.
(7) Clement, B.; Demesmaeker, M.; Linne, S. Microsomal catalyzed *N*-hydroxylation of guanabenz and reduction of the *N*-hydroxylated metabolite: characterization of the two reactions and genotoxic potential of guanoxabenz. *Chem. Res. Toxicol.* **1996**, *9*, 682–688.
(8) Kadlubar, F. F.; McKee, E. M.; Ziegler, D. M. Reduced pyridine nucleotide-dependent *N*-hydroxy amine oxidase and reductase activities of hepatic microsomes. *Arch. Biochem. Biophys.* **1973**, *156*, 46–57.
(9) Kadlubar, F. F.; Ziegler, D. M. Properties of a NADH-dependent *N*-hydroxy amine reductase isolated from pig liver microsomes. *Arch. Biochem. Biophys.* **1974**, *162*, 83–92.
(10) Bernheim, M. L.; Hochstein, P. Reduction of hydroxylamine by rat liver mitochondria. *Arch. Biochem. Biophys.* **1968**, *124*, 436–442.
(11) Bernheim, M. L. The hydroxylamine reductase of mitochondria. *Arch. Biochem. Biophys.* **1969**, *134*, 408–413.
(12) Bernheim, M. L. The reduction of hydroxylamine and some aryl hydroxamates by liver mitochondria from mammals and birds. *Enzymologia* **1972**, *43*, 167–176.
(13) Clement, B.; Lomb, R.; Möller, W. Isolation and characterization of the protein components of the liver microsomal O2-insensitive NADH-benzamidoxime reductase. *J. Biol. Chem.* **1997**, *272*, 19615–19620.
(14) Havemeyer, A.; Bittner, F.; Wollers, S.; Mendel, R.; Kunze, T.; Clement, B. Identification of the missing component in the mitochondrial benzamidoxime prodrug-converting system as a novel molybdenum enzyme. *J. Biol. Chem.* **2006**, *281*, 34796–34802.
(15) Nason, A.; Lee, K. Y.; Pan, S. S.; Ketchum, P. A.; Lamberti, A.; DeVries, J. In vitro formation of assimilatory reduced nicotinamide adenine dinucleotide phosphate: nitrate reductase from a *Neurospora* mutant and a component of molybdenum enzymes. *Proc. Natl. Acad. Sci. U.S.A* **1971**, *68*, 3242–3246.
(16) Andersson, S.; Hofmann, Y.; Nordling, A.; Li, X. Q.; Nivelius, S.; Andersson, T. B.; Ingelman-Sundberg, M.; Johansson, I. Characterization and partial purification of the rat and human enzyme systems active in the reduction of *N*-hydroxymelagatran and benzamidoxime. *Drug Metab. Dispos.* **2005**, *33*, 570–578.
(17) Kurian, J. R.; Bajad, S. U.; Miller, J. L.; Chin, N. A.; Trepanier, L. A. NADH cytochrome b5 reductase and cytochrome b5 catalyze the microsomal reduction of xenobiotic hydroxylamines and amidoximes in humans. *J. Pharmacol. Exp. Ther.* **2004**, *311*, 1171–1178.
(18) Kurian, J. R.; Chin, N. A.; Longlais, B. J.; Hayes, K. L.; Trepanier, L. A. Reductive detoxification of arylhydroxylamine carcinogens by human NADH cytochrome b5 reductase and cytochrome b5. *Chem. Res. Toxicol.* **2006**, *19*, 1366–1373.
(19) Saulter, J. Y.; Kurian, J. R.; Trepanier, L. A.; Tidwell, R. R.; Bridges, A. S.; Boykin, D. W.; Stephens, C. E.; Anbazhagan, M.; Hall, J. E. Unusual dehydroxylation of antimicrobial amidoxime prodrugs by cytochrome b5 and NADH cytochrome b5 reductase. *Drug Metab. Dispos.* **2005**, *33*, 1886–1893.
(20) Clement, B.; Behrens, D.; Amschler, J.; Matschke, K.; Wolf, S.; Havemeyer, A. Reduction of sulfamethoxazole and dapsone hydroxylamines by a microsomal enzyme system purified from pig liver and pig and human liver microsomes. *Life Sci.* **2005**, *77*, 205–219.
(21) Clement, B.; Mau, S.; Deters, S.; Havemeyer, A. Hepatic, extrahepatic, microsomal, and mitochondrial activation of the *N*-hydroxylated prodrugs benzamidoxime, guanoxabenz, and Ro 48-3656 ([[1-[(2*s*)-2-[[4-[(hydroxyamino)iminomethyl]benzoyl]amino]-1-oxopropyl]-4-piperid inyl]oxy]-acetic acid). *Drug Metab. Dispos.* **2005**, *33*, 1740–1747.
(22) Torres, R. A.; Korzekwa, K. R.; McMasters, D. R.; Fandozzi, C. M.; Jones, J. P. Use of density functional calculations to predict the regioselectivity of drugs and molecules metabolized by aldehyde oxidase. *J. Med. Chem.* **2007**, *50*, 4642–4647.
(23) Sergeev, G. V.; Gilep, A. A.; Estabrook, R. W.; Usanov, S. A. Expression of outer mitochondrial membrane cytochrome b5 in *Escherichia coli* purification of the recombinant protein and studies of its interaction with electron-transfer partners. *Biochemistry (Moscow)* **2006**, *71*, 790–799.
(24) Shirabe, K.; Landi, M. T.; Takeshita, M.; Uziel, G.; Fedrizzi, E.; Borgese, N. A novel point mutation in a 3′ splice site of the NADH-cytochrome b5 reductase gene results in immunologically undetectable enzyme and impaired NADH-dependent ascorbate regeneration in cultured fibroblasts of a patient with type II hereditary methemoglobinemia. *Am. J. Hum. Genet.* **1995**, *57*, 302–310.
(25) Wittke, B.; Mackie, I. J.; Machin, S. J.; Timm, U.; Zell, M.; Goggin, T. Pharmacokinetics and pharmacodynamics of Ro 44-3888 after single ascending oral doses of sibrafiban, an oral platelet aggregation inhibitor, in healthy male volunteers. *Br. J. Clin. Pharmacol.* **1999**, *47*, 521–530.
(26) Lanteri, C. A.; Trumpower, B. L.; Tidwell, R. R.; Meshnick, S. R. DB75, a novel trypanocidal agent, disrupts mitochondrial function in *Saccharomyces cerevisiae*. *Antimicrob. Agents Chemother.* **2004**, *48*, 3968–3974.
(27) Stuehr, D. J.; Kwon, N. S.; Nathan, C. F.; Griffith, O. W.; Feldman, P. L.; Wiseman, J. *N*-omega-Hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. *J. Biol. Chem.* **1991**, *266*, 6259–6263.
(28) Krüger, P. Ueber Abkömmlinge des Benzenylamidoxims. *Ber. Dtsch. Chem. Ges.* **1885**, *18*, 1055–1060.
(29) Clement, B.; Bürenheide, A.; Rieckert, W.; Schwarz, J. Diacetyldiamidoximeester of pentamidine, a prodrug for treatment of protozoal diseases: synthesis, in vitro and in vivo biotransformation. *ChemMedChem* **2006**, *1*, 1260–1267.
(30) Clement, B.; Immel, M.; Raether, W. Metabolic *N*-hydroxylation of diminazene in vitro. *Arzneimittelforschung* **1992**, *42*, 1497–1504.
(31) Bailey, D. M.; DeGrazia, C. G. Hydroxyguanidines. A new class of antihypertensive agents. *J. Med. Chem.* **1973**, *16*, 151–156.
(32) Palmer, T.; Santini, C. L.; IobbiNivol, C.; Eaves, D. J.; Boxer, D. H.; Giordano, G. Involvement of the narJ and mob gene products in distinct steps in the biosynthesis of the molybdoenzyme nitrate reductase in *Escherichia coli*. *Mol. Microbiol.* **1996**, *20*, 875–884.
(33) Johnson, J. L.; Rajagopalan, K. V. Structural and metabolic relationship between the molybdenum cofactor and urothione. *Proc. Natl. Acad. Sci. U.S.A* **1982**, *79*, 6856–6860.
(34) Schwarz, G.; Boxer, D. H.; Mendel, R. R. Molybdenum cofactor biosynthesis. The plant protein Cnx1 binds molybdopterin with high affinity. *J. Biol. Chem.* **1997**, *272*, 26811–26814.

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