Role of Tyrosine Residues in Hg(II) Detoxification by Mercuric Reductase from *Bacillus* sp. Strain RC607†

Denise Rennex,‡ Richard T. Cummings,§,⊥ Mark Pickett,‖ Christopher T. Walsh,§ and Mark Bradley*,‡

Department of Chemistry, University of Southampton, Southampton SO9 5NH, U.K., Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, and Department of Molecular Microbiology, University of Southampton Medical School, Southampton General Hospital, Tremona Road, Southampton SO9 4XY, U.K.

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ABSTRACT: Two tyrosine residues of mercuric reductase (MerA), Tyr-264 and Tyr-605, which were shown by the X-ray crystal structure to be involved in metal binding, were changed to phenylalanine residues by site-directed mutagenesis, both singly (Y264F, Y605F) and to form a double mutant (Y264,605F). The effect of these mutations on Hg(II) reduction activity varied. While MerA Y605F has a similar apparent K_m to the wild-type enzyme and an apparent k_{cat} reduced by 6-fold, MerA Y264F has an apparent K_m 5-fold lower than the wild type and apparent k_{cat} 160-fold lower. The double mutant MerA Y264,605F has the same apparent K_m as MerA Y264F, but its apparent k_{cat} was reduced by a further 7-fold. These results show that the roles of the two tyrosine residues are not equivalent and that Y264 is important for catalysis, possibly by destabilizing the binding of Hg(II) to the two ligating thiolates at the active site of MerA.

Mercuric reductase (MerA[1]), one of the proteins encoded by the mercury resistance (*mer*) operon, catalyzes a unique reaction in which mercuric ions Hg(II) are reduced to mercury metal Hg(0) using NADPH as a source of reducing power (Summers, 1986; Fox & Walsh, 1982). This enzyme has been purified and cloned from both Gram-negative and Gram-positive bacteria and shown to be a member of the family of flavin-dependent disulfide oxidoreductases, the parent of which is glutathione reductase (Brown et al., 1983; Laddaga et al., 1987; Fox & Walsh, 1982; Fox & Walsh, 1983). Like glutathione reductase, MerA is active as a homodimer and catalyzes the transfer of 2 electrons from NADPH via an enzyme-bound FAD into a disulfide bond. The chemistry then diverges among members of this family of enzymes. In glutathione reductase, the electrons flow first into the disulfide bond of oxidized glutathione, and then into a mixed disulfide bond formed between the enzyme and glutathione. In MerA, however, a second equivalent of NADPH is required to generate the 4-electron-reduced enzyme (EH_4 or EH_2–NADPH), and it is this form which catalyzes the rate-limiting reduction of the mercuric ion (Sahlman et al., 1984; Miller et al., 1989; Distelfano et al., 1989). In contrast to MerA, other enzymes of this family such as glutathione reductase and lipoamide dehydrogenase are inhibited by Hg(II), presumably due to the high affinity of mercuric ions for the catalytically essential thiol groups at the active site (Massey & Williams, 1965; Casola & Massey, 1966). The structural and redox cycling differences that exist between MerA and the other members of this family are undoubtedly responsible for the ability of MerA to change the effect of mercury binding from inhibition to catalysis and detoxification.

MerA has been purified from the Gram-positive *Bacillus* sp. strain RC607, and the *merA* gene encoding this protein has been cloned (Wang et al., 1989). The crystal structure of this protein has also been recently determined (Schiering et al., 1991). In addition to the core region (amino acid residues 167–616 of *Bacillus* MerA) which shows a very similar structure to that of human glutathione reductase, MerA has extensions at both the N- and C-termini relative to glutathione reductase. The N-terminal region (residues 1–166), which consists of two 80 amino acid repeats that show homology to the periplasmic Hg(II)-binding protein MerP (Wang et al., 1989), is not distinguishable in the X-ray structure of the protein due to its high mobility (Schiering et al., 1991). MerA encoded by Tn501 from *Pseudomonas aeruginosa* has only one of these repeats at its N-terminus, which can be cleaved from the protein without affecting enzyme activity (Fox & Walsh, 1982). The C-terminal extension of 15 amino acids contains a pair of cysteine residues (Cys-628 and Cys-629) that have been shown to be catalytically important by site-directed mutagenesis of the equivalent residues in Tn501 *merA* (Moore & Walsh, 1989). These cysteine residues, together with the active-site cysteines from the other subunit (Cys-207 and Cys-212 in *Bacillus* MerA) were originally proposed to form a binding site for Hg(II), in which the mercuric ion was ligated in either a tri- or tetradentate manner by the thiol groups of these residues (Miller et al., 1989). Linear, bidentate complexes of Hg(II) are commonly found with thiol ligands and have exceptionally high stability constants (Wright et al., 1990; Stricks & Koltholff, 1953). Since the rate at which the free cofactor $FADH_2$ reduces Hg(II) ions is inversely related to the stability constant of the complex of Hg(II) with a particular ligand (Cummings & Walsh, 1992; Distefano et al., 1989; Gopinath et al., 1989), a destabilized tri- or tetradentate coordination was suggested as a way in which

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* To whom correspondence should be addressed.

‡ University of Southampton.

§ Harvard Medical School.

⊥ Present address: Department of Biophysical Chemistry, MRL, P.O. Box 2000, Rahway, NJ 07065-0900.

‖ Southampton General Hospital.

[1] Abbreviations: bp, base pair; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; IPTG, isopropyl β-D-thiogalactopyranoside; kb, kilobase pair; MerA, mercuric reductase; $NADP^+$, β-nicotinamide adenine dinucleotide phosphate; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; PVDF, poly(vinylidene difluoride); RBS, ribosome binding site; thio-$NADP^+$, thionicotinamide adenine dinucleotide phosphate.

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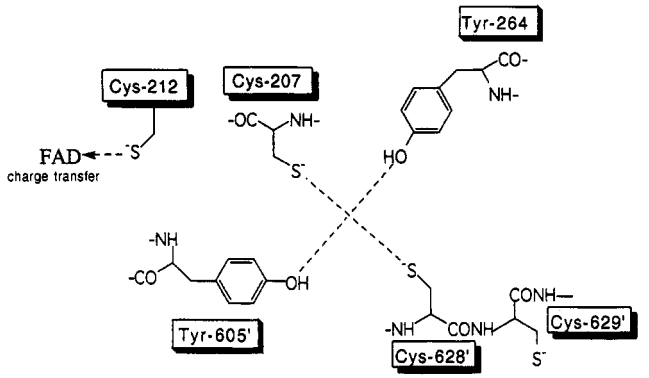

FIGURE 1: Schematic view of the active site of MerA reduced with NADPH showing the position of four residues proposed to form a binding site for Hg(II). These are Cys-207 and Tyr-264 from one subunit of the enzyme and Cys-628′ and Tyr-605′ from the other subunit. Cys-212 forms a charge transfer complex with the flavin ring, and Cys-629′ is oriented away from the active site. The lines connecting the hydroxyl groups of the two tyrosine residues and the sulfur atoms of the two cysteine residues are 1.3 Å offset from perpendicular, and the distorted tetrahedron formed by these ligands is the binding site of Cd(II) and possibly of Hg(II). The distance between the two sulfur atoms of Cys-207 and Cys-628′ is 5 Å, and the distance between the hydroxyl groups of Tyr-264 and Tyr-605′ is 5.8 Å (Schiering et al., 1991).

MerA could bind its substrate and effect catalysis without forming a dead-end complex (Miller et al., 1989). However, the X-ray crystal structure showed that, in the reduced form of the enzyme, the proposed binding site for Hg(II) was formed by only two thiol groups of Cys-207 and Cys-628′ and also by the hydroxyl groups of Tyr-264 and Tyr-605′ (Figure 1). The structure of the enzyme with the competitive inhibitor Cd(II) bound showed that this ion was bound in a distorted tetrahedron by these four groups with the bond distances S–M = 2.2 Å and O–M = 3.1 Å, an example of classic primary and secondary coordination (Grdenic, 1965).

The aim of the present work was to investigate the roles of the two tyrosine residues Tyr-264 and Tyr-605 in the reduction of Hg(II) by MerA. To this end, *Bacillus merA* was cloned into a suitable vehicle to allow mutagenesis and overexpression of MerA. The two residues Tyr-264 and Tyr-605 were changed by site-directed mutagenesis to phenylalanines, and three mutant proteins (Y264F, Y605F, and Y264F,Y605F) were expressed, purified, and characterized.

MATERIALS AND METHODS

Materials. RedA and OrangeA matrix gels and Centricon concentrators were from Amicon (Danvers, MA).

Cloning and Mutagenesis of merA. A 2.1-kb *Sac*II/*Hinc*II fragment from the plasmid pYW40, containing the *merA* gene of *Bacillus* sp. strain RC607 (Wang et al., 1989), was subcloned into Bluescript II SK(+). This was used as the basis for the preparation of all the mutants described below. Site-directed mutagenesis was performed by the method of Kunkel et al. (1987) using the *Escherichia coli* strain CJ236. An *Eco*RI site was generated 15 bp upstream of the start codon of *merA* using the oligonucleotide GTCGAAT-*TC*AAGGAGG, allowing the whole *merA* gene including its natural RBS to be excised as an *Eco*RI/*Hinc*II fragment. This was ligated into pUC18 previously cut with *Eco*RI and *Hinc*II, enabling an *Eco*RI/*Hind*III fragment containing the *merA* gene to be excised and inserted into two overexpression vectors, IBI30 (IBI, New Haven, CT) and pKK223-3 (Pharmacia, Piscataway, NJ), both previously cut with *Eco*RI and *Hind*III.

Two mutants of *merA*, Y264F and Y605F, were produced by single base changes from TAT (Tyr) to TTT (Phe), using the oligonucleotides GCGAAATGAAAAA*TTT*GTGAATT-TAA (Y264F) and CGATGGCTCCA*TTT*CTAACAATG (Y605F). A single *Cla*I site, which was situated between the two sites of mutation and 1545 bp from the beginning of the start codon, was used to generate a *Cla*I/*Hinc*II fragment from *merA* Y605F, and the double mutant *merA* Y264,605F was constructed by replacing the *Cla*I/*Hinc*II fragment of *merA* Y264F with this fragment. The wild-type and mutant *merA* genes were sequenced in their entirety using a single-stranded DNA template generated using the helper phage VCSM13 and 10 short synthetic oligonucleotides. This was done either manually by the method of Sanger et al. (1977) using Sequenase (United States Biochemical Corp., Cleveland, OH) or by *Taq* cycle DNA sequencing utilizing an Applied Biosystems 373A DNA sequencer and fluorescent dideoxy-nucleotides.

A single *Xma*I site, which was located upstream of the two mutation sites in the *merA* gene at a position 192 bp downstream from the start codon, was used to produce *Xma*I/*Hinc*II fragments of the Y264F, Y605F, and Y264,605F *merA* genes. These fragments were cloned into the IBI30–*merA* overexpression system under the control of the T7 RNA polymerase promoter by replacing the *Xma*I/*Hinc*II fragment of the wild-type gene in this vector with the fragments of the mutants. Each of the *merA* genes was also cut from the appropriate IBI30 construct using *Eco*RI and *Hind*III and inserted into pKK223-3 to place the *merA* gene under the control of the *tac* promoter.

MerA protein was expressed in *E. coli* strain W3110 containing the heat shock T7 system plasmid pGP1–2 (Tabor & Richardson, 1985) and IBI30–*merA*. Cultures were grown at 30 °C in 2xTY media supplemented with 100 μg/mL ampicillin and 100 μg/mL kanamycin until an OD_{600} of 1.0–1.2 was reached. Expression was induced by heating at 43 °C for 30 min, and growth was then continued at 37 °C for a further 2–3 h. MerA protein was also expressed using the pKK223-3 vector containing the *tac* promoter in *E. coli* strain XA90. Cultures were grown at 37 °C in 2× TY media which was supplemented with 100 μg/mL ampicillin until an OD_{600} of about 0.5 was reached. Expression was induced by the addition of IPTG to a final concentration of 0.2 mM, and the cells were then grown a further 4–5 h at 37 °C.

Purification of MerA. Wild-type and mutant MerA proteins were purified essentially as described by Wang et al. (1989) except that cells were lysed by treatment with lysozyme (0.3 mg/mL) followed by sonication, and the buffer used during purification was 25 mM potassium phosphate (pH 7.5) containing 0.5 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. Active fractions were pooled and concentrated if necessary, by centrifugation in a Centricon microconcentrator. Tightly-bound $NADP^+$ was removed by dialysis against 25 mM potassium phosphate buffer (pH 7.5) containing 0.5 mM EDTA and 2 M KBr (Fox & Walsh, 1982) and then dialyzed to remove KBr against 25 mM potassium phosphate buffer (pH 7.5) containing 0.5 mM EDTA.

SDS–polyacrylamide gel electrophoresis was carried out by the method of Laemmli (1970). Purified MerA was blotted onto PVDF membrane and sequenced using an Applied Biosystems 477A protein sequencer by the protein sequencing facility at the Department of Biochemistry, University of Southampton.

Assay for MerA Activity and Kinetic Analysis. MerA activity was assayed at 25 °C in 50 mM potassium phosphate

buffer (pH 7.5) containing 1 mM EDTA, 46 μM NADPH, 2 mM 2-mercaptoethanol, and 100 μM $HgCl_2$. The protein concentration was either measured by the method of Bradford (1976) using bovine serum albumin as a standard or determined from the absorbance of the flavin prosthetic group at 458 nm (ϵ_{458} = 11.2 mM^{-1} cm^{-1}), assuming one flavin per MerA monomer. The concentrations of Hg(II) were determined by end point titrations using NADPH and wild-type MerA assuming an ϵ_{340} for NADPH of 6.22 mM^{-1} cm^{-1}. Kinetic analysis of Hg(II) reduction was performed as described above but with varying concentrations of $HgCl_2$ and without EDTA.

Transhydrogenation activity was measured at 25 °C in 50 mM potassium phosphate buffer (pH 7.5) containing 100 μM thio-$NADP^+$ and varying concentrations of NADPH.

Initial rate data were analyzed by Eadie-Hofstee plots using the EnzymeKinetics program (Trinity Software, Campton, NH). Kinetic constants were calculated per monomer of MerA enzyme.

O_2 consumption was measured with a Rank Brothers Ltd. (Cambridge) O_2 electrode at 23 °C in 50 mM potassium phosphate buffer (pH 7.5) containing 92 μM NADPH, 2 mM 2-mercaptoethanol, and 20 μM $HgCl_2$. The concentrations of enzyme used were 190 and 82 nM for MerA Y264F and MerA Y264,605F, respectively.

Spectrophotometry. All UV/vis spectra were recorded with a Cary 1 spectrophotometer.

RESULTS AND DISCUSSION

Wild-type *Bacillus* MerA expressed in *E. coli*, using either the T7 heat shock or pKK223-3 expression system, was identical to the enzyme previously isolated from *Bacillus* RC607 (Wang et al., 1989). It could be purified by binding to Red A matrix gel, did not bind to either Orange A matrix gel or 2′,5′-ADP–Sepharose, and SDS–polyacrylamide gel electrophoresis of the purified protein showed a predominant band of 69 kDa (data not shown). The enzyme had a pH optimum of 7.0, an apparent K_m for Hg(II) of 30 μM, and apparent k_{cat} of 12 s^{-1}. These values agree well with the K_m for Hg(II) of 15 μM and k_{cat} of 19 s^{-1} previously reported (Wang et al., 1989). The N-terminal sequence (Met-Lys-Lys-Tyr-Arg-Val-Asn-Val-Gln-Gly) was identical to that previously reported for the *Bacillus* enzyme (Wang et al., 1989). Yields of purified MerA protein using either the IBI30 or pKK223–3 expression system ranged between 1 and 2 mg/L of culture.

All three mutant enzymes, MerA Y264F, Y605F, and Y264,605F, were purified in an identical manner to the wild-type enzyme, and all four enzymes had the same apparent size by SDS–polyacrylamide gel electrophoresis (data not shown). Members of the family of flavin-dependent disulfide oxidoreductases are able to catalyze the reduction of thio-$NADP^+$ by NADPH, which is believed to involve the reduction and reoxidation of the enzyme-bound flavin (Schultz et al., 1985). Kinetic analysis of this activity of the wild-type and the three mutant enzymes showed minimal changes in transhydrogenation activity that did not correlate with the effect of the mutations on Hg(II) reductase activity (Table I). The ability of the three mutant proteins to bind NADPH, to reduce thio-$NADP^+$, and to be purified by affinity chromatography in a manner identical to that of the wild-type enzyme suggests that the mutations that were made did not alter either the tertiary structure or the general properties of the mutant enzymes and that alterations in the NADPH-binding domain of the mutant enzymes are not responsible for the changes in Hg(II) reductase activity.

Table I: Kinetic Constants for Reduction of Hg(II) and Transhydrogenation of Thio-$NADP^+$ by Wild-Type and Mutant MerA Enzymes[a]

enzyme	K_m[Hg(II)] (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1}) (transhydrog)	K_m[NADPH] (μM) (transhydrog)
wild type	30	12	4.0×10^5	1.3	0.3
Y264F	6	0.073	1.2×10^4	1.8	2.9
Y605F	39	1.9	4.9×10^4	0.9	0.6
Y264,605F	6	0.011	1.8×10^3	1.2	1.4

[a] Values of k_{cat} and K_m are apparent values, as in each case only one substrate was varied.

The results of kinetic analysis of Hg(II) reduction by wild-type and mutant enzymes are shown in Table I. True kinetic constants for Hg(II) reduction activity could not be determined, as the rate of reaction at constant Hg(II) concentration did not vary until the concentration of NADPH was reduced to 2.5 μM. All experiments were therefore performed with an apparently saturating NADPH concentration of 46 μM, and K_m and k_{cat} are thus apparent values. MerA Y605F was altered very little from the wild-type protein, the K_m for Hg(II) remained about the same, and k_{cat} was reduced by only about 6-fold. Due to the high background oxidase rate of the mutant proteins MerA Y264F and Y264,605F directly after elution from the RedA matrix gel column, it was essential that the kinetic data for these enzymes were obtained using the KBr-treated proteins. Treatment of wild-type MerA and MerA Y605F with KBr did not alter the K_m for Hg(II) of either of these enzymes and did not alter k_{cat} of MerA Y605F, although k_{cat} of the wild-type enzyme decreased to 3 s^{-1}. A similar effect of dialysis against solutions containing 2 M KBr on wild-type MerA from Tn501 has previously been reported by Schultz et al. (1985). The results for the mutant Y264F show a very dramatic alteration in catalytic activity. The k_{cat} is reduced by 160-fold, and the K_m for Hg(II) falls by 5-fold, with a resulting decrease in overall catalytic efficiency from 4.0×10^5 to 1.2×10^4 s^{-1} M^{-1}. The double mutant shows the most impaired catalytic activity; k_{cat} is reduced by a factor of approximately 1000-fold compared with the wild type, with a turnover number now of only 0.011 s^{-1}. The K_m for Hg(II) of the double mutant remains the same as that of MerA Y264F, and the catalytic efficiency of 1.8×10^3 s^{-1} M^{-1} is 220-fold lower than that of the wild-type enzyme. It has been previously reported that the observed rate of Hg(II)-induced NADPH consumption of C135S mutants of Tn501 MerA is due to an increase in the oxidase activity of these enzymes (Distefano et al., 1989). To ensure that the low rates of NADPH consumption of MerA Y264F and MerA Y264,605F were due to Hg(II) reduction, the oxidase rates of these two enzymes were measured with an O_2 electrode. In both cases, addition of Hg(II) did not result in increased O_2 consumption above the background. The low rates of activity observed are thus due to Hg(II) reduction activity.

It appears that mutations of each of the two tyrosine residues to phenylalanines have very different effects on the Hg(II) reductase activity of MerA. While the removal of the hydroxyl group of Tyr-605 results in only a 6-fold decrease in the rate of the enzyme, removal of that of Tyr-264 results in a decrease in the K_m for Hg(II) and a greatly impaired turnover number. The results of the double mutant show that the effects of the two mutations are essentially additive in terms of changes in the transition-state stabilization energies ($\Delta\Delta G_T$) (Wells, 1990).

These results demonstrate that although the X-ray crystallographic data point toward a tetracoordinate binding site for Hg(II) within the active site of MerA, the enzyme appears to function perfectly adequately with the removal of one of them (Tyr-605), consistent with functional tridentate Hg(II) ligation. However, the two tyrosine ligands are obviously not identical in function since removal of Tyr-264 causes a marked decrease in the k_{cat} for Hg(II) reduction. From the study of the uncatalyzed reduction of Hg(II) by reduced flavins, it has been shown that the rate of reaction decreases with increasing stability of the Hg(II)–ligand complex (Cummings & Walsh, 1992; Distefano et al., 1989; Gopinath et al., 1989). It has been postulated that one of the roles of the tyrosine residues is to provide an influence to weaken the binding of the mercuric ion by the thiolates (Cys-207 and Cys-628′), the presence of which might otherwise lead to irreversible binding of Hg(II) and formation of a dead-end complex (Miller et al., 1989; Petsko, 1991). This proposition is supported by the double mutant Y264,605F. In this enzyme the turnover number is reduced to a mere 0.011 s^{-1}.

Since *Bacillus* MerA crystallizes, some of these questions will be investigated further by X-ray analysis of the mutant proteins. Given the modest effect of the mutation Y605F, it is interesting to note that this residue is in the equivalent position in MerA as the catalytically essential His467 that acts as a proton acceptor/donor in human glutathione reductase (Karplus & Schulz, 1989). It is possible that the presence of a tyrosine residue in this position rather than a histidine residue (which is only one base change from CA(C/T) for histidine to TA(C/T) for tyrosine) results in the switch from a strongly coordinating imidazole side chain that is a common ligand for metal ions in metal-dependent enzymes (Glusker, 1992) and which is known to bind Hg(II) in cysteine and histidine-containing peptides (Adachi et al., 1992) to the more weakly coordinating phenol side chain of tyrosine. This residue switch may be one of the keys in the conversion of a disulfide reductase catalyst to a Hg(II) reductase by active site reengineering to bind Hg(II) just loosely enough to be able to reduce it.

The *Bacillus merA* gene is now available in a form suitable for mutagenesis and in expression systems that will enable the production of sufficient protein for crystallography purposes. Further analyses of the mechanism of this unique enzyme will include the mutation of Cys-212, which forms a charge transfer complex with the flavin ring (Schiering et al., 1991). Mutants of MerA at this position are 10^3–10^4 less active than the wild-type enzyme (Distefano et al., 1989) but retain the residues implicated in Hg(II)-binding, which should allow the determination of the crystal structure of MerA with Hg(II) bound at the active site.

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