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Interaction of Tn501 Mercuric Reductase and Dihydroflavin Adenine Dinucleotide Anion with Metal Ions: Implications for the Mechanism of Mercuric Reductase Mediated Hg(II) Reduction†

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ABSTRACT: The flavoprotein Tn501 mercuric reductase (MerA) catalyzes the reduction of Hg(II) to Hg(0) through the intermediacy of the tightly bound two-electron-reduced cofactor $FADH^-$. To gain insight into the MerA mechanism, the interaction of the holoenzyme or free $FADH^-$ with various metal ions was investigated. The free two-electron-reduced FAD cofactor, $FADH^-$, readily reduces a variety of metal ions, provided they have suitably high redox potentials. For Hg(II) with various ligands, the rate of reduction is inversely proportional to the stability of the Hg(II)-ligand complex. These results are consistent with the free cofactor reducing metal ions by an outer-sphere electron transfer mechanism. In contrast, MerA can tightly bind several redox labile metal ions, but only Hg(II) is reduced. The inability of MerA to reduce these bound metal ions may suggest that MerA differs from free $FADH^-$ and utilizes an inner-sphere electron transfer mechanism in Hg(II) reduction.

Cellular functions and enzymatic activities are often dependent on critical thiols such as cysteine (Cys) residues in proteins and glutathione. Consequently, organomercurials and mercuric ion are highly toxic to living systems by virtue of tight binding to sulfur [at physiological pH, K_f for $Hg(Cys)_2 > 10^{40.3}$ M^{-2} (Stricks & Koltholff, 1953)]. In contrast, metallic mercury displays no propensity to bind thiols and is much less toxic. Against this background, bacteria have evolved a unique detoxification mechanism for mercury compounds (Moore et al., 1990). Rather than ion removal by sequestration or active

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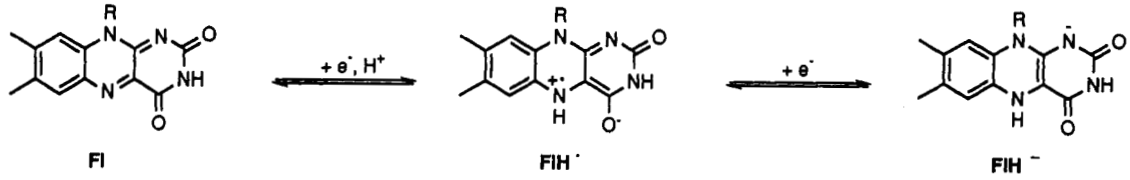
Scheme I



FAD: R = Adenine Dinucleotide

transport, the final step in mercury detoxification is the reduction of Hg(II) to Hg(0). The nontoxic metal thus produced exits the cell by passive diffusion or volatilization.

The flavoprotein mercuric reductase (MerA)[1] catalyzes the NADPH-dependent reduction of Hg(II) to Hg(0):

$$Hg(SR)_2 + NADPH + H^+ \rightarrow Hg(0) + NADP^+ + 2\ RSH$$

The most thoroughly studied mercuric reductase, that isolated from *Pseudomonas aeruginosa* (Tn501 MerA), consists of 561 amino acids and, in its active form, is an α_2 homodimer (118 kDa) with two active sites per dimer (Brown et al., 1983; Fox & Walsh, 1982).[2] It is a member of the disulfide oxidoreductase class of enzymes, other members of which include lipoamide dehydrogenase, glutathione reductase, trypanothione reductase, and thioredoxin reductase. All members of this family are thought to have a similar active site consisting of a nicotinamide-binding site, a noncovalently associated flavin, and a redox-active disulfide (Williams, 1991). Nevertheless, mercuric reductase is unique in its ability to reduce Hg(II) at a catalytically viable rate. Mercury compounds are strongly deleterious to other disulfide oxidoreductases. For example, yeast glutathione reductase reduces Hg(II) at only 0.01% the rate of MerA (Moore & Walsh, 1989), and its own enzymatic activity is strongly inhibited by phenyl mercuric acetate (Massey & Williams, 1965).

Mercuric reductase diverges structurally from other disulfide oxidoreductases. While other family members have a single set of paired cysteines (the redox-active disulfide), Tn501 MerA contains three disulfides: $Cys_{10}Cys_{13}$, $Cys_{135}Cys_{140}$, and $Cys_{558}Cys_{559}$. $Cys_{135}Cys_{140}$ is the redox-active disulfide (Cys_{140} proximal to the flavin). The N-terminal pair is dispensable, removal of the first 85 amino acids of Tn501 MerA by treatment with α-chymotrypsin caused no change in catalytic activity (Fox & Walsh, 1983), and site-directed mutagenesis of $Cys_{10}Cys_{13}$ to $Ala_{10}Ala_{13}$ did not alter either in vitro specific activity (Miller et al., 1989) or in vivo Hg(II) resistance (Moore & Walsh, 1989). In contrast, the C-terminal pair, $Cys_{558}Cys_{559}$, is critical for enzymatic activity, with the site-directed double mutant $Ala_{558}Ala_{559}$ having only 0.09% in vitro wild-type activity (Moore & Walsh, 1989).[3] This auxiliary dithiol is conserved in all mercuric reductases sequenced to date and is part of the active site, with the two cysteine pairs being donated from different chains of the α_2 homodimer (Distefano et al., 1990; Miller et al., 1989).

The redox-active portion of FAD and its derivatives is the flavin ring system. This ring system can assume several redox states, specifically the oxidized flavin (Fl_{ox}), the one-electron-reduced semiquinone state ($FlH^{\cdot}$), and the two-electron-reduced dihydroflavin (FlH_2). The predominant forms of these different redox states at physiological pH are illustrated in Scheme I. The two-electron-reduced cofactor is also found as the monoanion $FADH^-$ [pK_a ($N-1$) = 6.2] (Hemmerich & Lauterwein, 1973).

The participation of reduced flavins in redox chemistry with metal ions was shown by reports that riboflavin semiquinone (Singh et al., 1982a) and dihydroriboflavin (Singh et al., 1982b) underwent one-electron transfers with metal ion oxidants. Subsequently, the one-electron reduction of Mn(III) porphyrin by a two-electron-reduced flavin in a flavin–porphyrin adduct was reported (Takeda et al., 1987). In work that is particularly relevant to MerA, it was demonstrated that $FADH^-$ and other dihydroflavin species reduced Hg(II) to Hg(0) (Distefano et al., 1989; Gopinath et al., 1989). While this last result clearly showed that dihydroflavin species could participate in net two-electron redox chemistry with Hg(II), its applicability to other two-electron-reducible metal ions was unknown. In this context, studies were undertaken to determine to what extent the $FADH^-$ cofactor participates in redox reactions with other metal ions.

The extent to which MerA can bind and reduce other metal ions has not been investigated extensively. In the only study which addressed this issue, Rinderle and co-workers reported that *Escherichia coli* R100 MerA could not reduce any metal ions except mercuric ions (Rinderle et al., 1983). However, several ions [Cd(II), Cu(II), Ag(I), Au(III)] did function as inhibitors. The K_i's were in the range of 1–80 μM, similar in magnitude to the K_m for Hg(II), 14 μM. Since that report, substantial insight into the MerA active site has been gained, in large part due to the overproduction of wild-type and mutant Tn501 MerA's and the application of the very sensitive anaerobic single-turnover assay (Williams et al., 1979) to MerA (Distefano et al., 1989). Nevertheless, the precise mechanism of MerA-mediated Hg(II) reduction, and the roles of the four active site thiols during the course of it, remain unknown. As a complementary approach to site-directed mutagenesis, the interaction of Tn501 MerA with several metal ions other than Hg(II) is now reported here.

Materials and Methods

Materials

$KAuBr_4$ (Matthey Chemicals, Hertfordshire, England),

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; MerA, mercuric reductase; E_{ox}, oxidized form of mercuric reductase; EH_2, mercuric reductase reduced with two electrons per monomer; EH_4, mercuric reductase reduced with four electrons per monomer; FAD, oxidized flavin adenine dinucleotide; $FADH^-$, dihydroflavin adenine dinucleotide monoanion; $NADP^+$, β-nicotinamide adenine dinucleotide phosphate; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; K_f, absolute stability (formation) constant for a complex; K_f^{app}, stability (formation) constant for a complex at a specific pH; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DMPS, 2,3-dimercaptopropylsulfonate; MES, 2-mercaptoethylsulfonate; SPLF, 3-(3-sulfopropyl)lumiflavin.

[2] Mercuric reductases have been identified and sequenced from several sources including both Gram negative and Gram positive bacteria (Moore et al., 1990). Results discussed here will, unless otherwise noted, be derived from experiments on Tn501 MerA.

[3] In the reductive environment of the cell, the critical C-terminal dithiol/disulfide Cys_{558}–Cys_{559} is presumably found in the active dithiol form. For in vitro studies, this pair must also be reduced for enzymatic activity [see Miller et al. (1989)].

$Cd(OAc)_2$ (Matheson, Coleman & Bell), $(NH_4)_2PtCl_4$ (Aldrich), $PdCl_2$ (Fisher), $HgCl_2$ (Alfa), $AgNO_3$ (Mallinckrodt), $CuCl_2$ (Sigma), $CoCl_2$ (Mallinkrodt), and $Fe(NO_3)_3$ (J. T. Baker) were used without further purification. CH_3HgCl (Alfa) was recrystallized from ethanol prior to use. FAD, NADPH, MES, DMPS, and sodium dithionite were all from Sigma and were used without further purification. β-mercaptoethanol was from Bio-Rad. HEPES and CHES buffers were obtained from United States Biochemical and Sigma, respectively. Orange-A Matrex was purchased from Amicon Corp., and P6-DG was from Bio-Rad.

Methods

Spectrophotometry. UV/vis spectra were recorded with either a Hewlett-Packard 8452A or a Gilford 260 spectrophotometer.

Protein Purification. Wild-type MerA was expressed from the overproduction plasmid pMD01 in *E. coli* W3110 $LacI^q$ and purified as described previously (Distefano et al., 1989). To prepare "clipped" enzyme, samples were digested with α-chymotrypsin, purified by chromatography with Orange-A, dialyzed against 3 M KBr and 100 mM sodium phosphate (pH 7.4) to remove any bound $NADP^+$, and then dialyzed against buffer without KBr to remove the salt (Fox & Walsh, 1982). Protein purity was determined using SDS–PAGE with a Pharmacia PhastSystem. Purified "clipped" protein samples were stored as ammonium sulfate pellets. Protein concentrations were determined by measuring the absorbance at 458 nm (ϵ_{458} = 11.3 $mmol^{-1}$ cm^{-1}). All enzyme concentrations are reported as *the concentration of active sites* (two active sites per MerA dimer).

To prepare samples for experiments, ammonium sulfate pellets were dissolved in 100 mM sodium phosphate buffer (pH 7.4) at a concentration of 0.5–2.0 mg/mL and exchanged into the desired buffer by two passages through P-6DG desalting gel (Bio-Rad). Once prepared, samples were stored at 4 °C until use (typically 1–5 days).

Aerobic Hg(II) Reduction. The ability of wild-type MerA to catalyze the $Hg(SR)_2$-dependent oxidation of NADPH was studied in several buffers systems employed in experiments described below in the manner described previously (Fox & Walsh, 1982). The relative rates of NADPH oxidation in the various buffers systems were found to be as follows (relative rate in parantheses): 80 mM potassium phosphate, pH 7.4 (100); 100 mM HEPES, pH 7.4 (110); 100 mM HEPES, pH 8.0 (57); and 100 mM CHES, pH 9.0 (70).

Preparation of Anaerobic Samples for Spectroscopy. Anaerobic spectroscopy was carried out in double-side-armed anaerobic cuvettes equipped with microliter syringes (Williams et al., 1979). Enzyme samples were prepared by placing them in the cuvettes (typically 1–2 mL), which were then made anaerobic by three repeated cycles of evacuation and purging with argon (which has been passed through an O_2 scrubber). Under such conditions there was no evidence of decomposition by spectroscopy, and buffer losses were small (<3%).

For the solutions containing free FAD cofactor, anaerobicity was achieved by bubbling argon directly through the solution for a period of 10–15 min. Similarly, stock solutions of sodium dithionite and the various metal ions were prepared in Wheaton vials and made anaerobic by bubbling argon through the samples. Again, buffer losses amounted to less than 3%. Sodium dithionite solutions were typically 1–5 mM. Solutions were prepared daily.

$FADH^-$-Mediated Reduction of Metal Ions. A solution of 80 μM FAD (1 mL) in 100 mM HEPES (pH 7.4) was placed into an anaerobic cuvette, made anaerobic, and then equilibrated in the spectrophotometer for 15 min. The sample was then titrated with sodium dithionite to 80–90% reduction as determined by the FAD maximal absorbance (450 nm) and subsequently monitored at 450 nm for 15 min to determine that a stable base line had been achieved. To this was added via microliter syringe 100 μL of a stock solution of the metal ion (16 mM, ≈20 equiv), and the solution was briefly mixed by tipping the cuvette. The reaction was monitored by observing the increase in A_{450} as FAD forms. Oxidations were followed until at least 97% reaction had occurred (>5 half-lives). Pseudo-first-order rate constants were then determined from a linear least-squares fit of the reaction trace. The background rate was determined by monitoring the anaerobic $FADH^-$ solution for several hours at 30 °C and was determined to be 0.00172 min^{-1}.

All stock metal ion solutions except those of Pd(II) were prepared in H_2O. $PdCl_2$ was solubilized in 1 M NaCl. Several ions (Table II) were only partially soluble and were solubilized by addition of EDTA (1–5 equiv). All stock solutions were prepared just prior to being made anaerobic.

The data for the Arrhenius plot of the $FADH^-$-mediated reduction of Hg–EDTA were obtained as described above in 100 mM HEPES (pH 7.4) with 90 μM FAD_{ox}, 1 mM $HgCl_2$, and 2 mM EDTA. Pseudo-first-order rate constants were determined at 5 °C intervals from 10 to 40 °C with five trials at each temperature. The average percent variance, *S*, of the data from the mean was ±4.3%. The uncertainty in the calculated activation parameters reflects this variance.

Biomolecular rate constants for the $PdCl_4^{2-}$ and Hg(II)–EDTA reductions were determined from reactions carried out in 100 mM HEPES (pH 7.4) with 90 μM FAD at 30 °C. For the Hg(II)–EDTA experiment, the ratio of $HgCl_2$ to EDTA was maintained at 2.00, and its concentration was varied as 0.5, 1.0, 1.5, and 2.0 mM. For the $PdCl_4^{2-}$ determination, the metal ion concentration was varied as 0.5, 0.75, 1.0, and 1.50 mM. For both the experiments, three trials were performed for each concentration and the average percent variance, *S*, of the data from the mean was <10%. The data from each experiment were linear ($R > 0.995$, $N = 4$).

Enzyme-Mediated Reduction of Alternate Metal Ions. Protein samples (1 mL of [MerA] = 25–50 μM in 100 mM HEPES, pH 7.4) were made anaerobic and equilibrated in the spectrophotometer for 15 min at 30 °C. The samples were then titrated with sodium dithionite until A_{560} had fallen to approximately 20% its maximal value. The background was then measured for 15 min, and, subsequent to this, an excess (3–20 equiv) of the metal ion (same form as that given in Table I) was added via microliter syringe (35–100 μL). Potential reoxidation was monitored at 458 nm for 30–120 min, although in no instance was EH_4 oxidation detected. To demonstrate that the enzyme was still viable, $HgCl_2$ (3 equiv) was then added and flavin oxidation observed.

Binding of Metal Ions to the MerA Active Site. Protein samples (2 mL of [MerA] = 30 μM in 100 mM CHES, pH 9.0) were made anaerobic and equilibrated in the spectrophotometer for 15 min at 30 °C. These samples were titrated with sodium dithionite until A_{458} had decreased to 55–65% of its initial value (≈3:1 mixture of EH_2/EH_4 MerA) and monitored for 10–15 min to verify that equilibrium had been reached. Metal ions [same form as that given in Table I, except Cd(II)] were then titrated in by addition of 10-μL aliquots of 2.92 mM solutions of the ions to a total addition of 100 μL (≈5 equiv of metal ion). Samples were allowed to equilibrate for 2 min before spectra were recorded for each point. A control (addition of H_2O) was carried out in an

Table I: Pseudo-First-Order Rate Constants for Hg(II)-Induced Flavin Oxidation

flavin	ligand	K_f[a]	k' (min^{-1})	relative rate	conditions	reference
FAD	Cl^-	10^{66} [b]	1.10	3700	pH 7.4, 10 °C	this work
FAD	EDTA	$10^{18.9}$	0.021	70	pH 7.4, 10 °C	this work
FAD	EDTA	$10^{18.9}$	0.120	400	pH 7.4, 30 °C	this work
SPLF[c]	MES[c]	$(10^{24.8})$[d]	0.019[e]	63	pH 4.7, 30 °C	Gopinath et al. (1989)
SPLF[c]	DMPS[c]	$10^{33.6}$	<0.0003	<1	pH 4.7, 30 °C	Gopinath et al. (1989)
MerA		$10^{39.8}$	780	2.6×10^6	pH 7.3, 25 °C	Sandstrom & Lindskog (1987)

[a] Apparent formation constant at the indicated pH. For the chloride ligands the units are M^{-2}, for all others M^{-1}. [b] Cotton and Wilkinson (1966). [c] SPLF, 3-(3-sulfopropyl)lumiflavin; MES, 2-mercaptoethylsulfonate; DMPS, 2,3-dimercaptopropylsulfonate. [d] Formation constant determined for penicillamine. [e] Predicted pseudo-first-order rate constant at [MES]/[Hg(II)] = 3.00.

identical manner and found to give a background rate of 8% quenching per 10 μL.

For the Cd(II) determinations, three buffer systems were used: 100 mM HEPES (pH 7.4, pH 8.0) and 100 mM CHES (pH 9.0). For these titrations [MerA] = 80 μM (1 mL), $[Cd(OAc)_2]$ = 20 mM, and the metal ion was titrated in increments of 1 equiv for a total of 6 equiv.

An additional experiment with MerA and $KAuBr_4$ in 100 mM CHES, pH 9.0, was performed. The anaerobic enzyme (50 μM, 1 mL) was titrated with sodium dithionite until it was an EH_2/EH_4 mixture of 76:24 as judged by UV–vis spectroscopy. To this was added $KAuBr_4$ (5 mM, 30 μL, 3 equiv) as a single addition, and, after 2 min, the spectrum was recorded.

Results and Discussion

Reduction of Hg(II) by FADH⁻. The ability of free $FADH^-$ (Distefano et al., 1989) or the flavin analogue 3-(3-sulfopropyl)lumiflavin (Gopinath et al., 1989) to reduce Hg(II) to Hg(0) serve as important chemical models for the enzymatic reduction. Additionally, the rate of the reduction depends on the nature of the ligand (Distefano et al., 1989; Gopinath et al., 1989). The more strongly associated ligands (higher K_f's) stabilize the Hg(II) ion, slowing the rate of reduction. The extent to which this is true for Hg(II) and other metal ions was examined further.

The free cofactor $FADH^-$, in the presence of excess Hg(II)–EDTA, reduces Hg(II) to Hg(0) with a pseudo-first-order rate constant of 0.30 min^{-1} at 37 °C in 100 mM phosphate buffer, pH 7.5 (Distefano et al., 1989). Instead of employing phosphate buffer, in which other metallic ions were only sparingly soluble, 100 mM HEPES, pH 7.4, has been utilized for these reported studies. Under anaerobic conditions, free FAD was reduced with sodium dithionite until the solution consisted of an approximately 80:20 mixture of $FADH^-$/FAD. A 20-fold excess of Hg(II)–EDTA was then added. Subsequently, the reoxidation of $FADH^-$ was followed by monitoring the formation of FAD (increase in A_{450}), and, from the resulting curve, a pseudo-first-order rate constant was determined. The rate of reduction was found to be 0.021 min^{-1} at 10 °C and 0.145 min^{-1} at 35 °C versus the 0.30 min^{-1} previously determined (Distefano et al., 1989). Reduction of $HgCl_2$ by $FADH^-$ at 10 °C occurred with a pseudo-first-order rate constant of 1.10 min^{-1} a rate acceleration of 53 over that observed for the Hg(II)–EDTA complex.

Rate constants for two-electron-reduced flavin mediated Hg(II) reduction where Hg(II) is stabilized by a variety of ligands are collected in Table I. Our results corroborate those of others (Gopinath et al., 1989), with the rate of reduction being inversely proportional to the stability of the corresponding Hg(II) complex. The apparent formation constant for MES could not be calculated as the absolute formation constant has not been reported; for this ligand, the value given for the structurally similar *N*-acetyl-D,L-penicillamine [K_f = $10^{35.4}$ M^{-2} (Casas & Jones, 1980)] was used. As demonstrated by Bruice and co-workers, the rate of reduction in the presence of the dithiol ligand DMPS is extremely slow: they were able to assign only a lower limit of $<3 \times 10^{-4}$ min^{-1} (Gopinath et al., 1989). The slower rate of Hg(II) reduction for the dithiol DMPS as compared to the monothiol MES was ascribed to the greater number of thiols proximal to the mercuric ion.[4] In this context, MerA appears paradoxical since mutational analysis (Moore et al., 1990) and the *Bacillus* MerA X-ray crystal structure (Scheiring et al., 1990, 1991) strongly suggest that at least three cysteine side chains are in the vicinity of Hg(II) bound in the active stie. As shown in Table I, the MerA-mediated reduction is very facile with a rate enhancement of $>10^6$ as compared to the dihydroflavin-mediated reduction of DMPS-ligated Hg(II). The apparent K_f for MerA was calculated from the K_m value reported by Sandstrom and Lindskog (1987) and indicates that the MerA active site has, as expected, a substantial affinity for the Hg(II) ion. Attempts to determine a K_f for MerA by competition experiments with other thiol ligands were unsuccessful (vide infra).

Variation in the Hg(II) reduction rate with temperature was also studied. The pseudo-first-order rate constant for the reaction of Hg–EDTA with $FADH^-$ was determined at 5 °C intervals from 10–40 °C. Graphing the data as an Arrhenius plot gave a straight line (R = 0.983, data not shown) from which an activation enthalpy, $\Delta H^{\ddagger}$ = 11.6 ± 0.5 kcal/mol, and activation entropy, $\Delta S^{\ddagger}$ = −26.3 ± 1.1 eu, were determined. The free energy of activation for the Hg(II)–EDTA reaction ($\Delta G^{\ddagger}$ = 20.0 ± 0.9 kcal/mol at 25 °C) probably derives, in large part, from the strong complexation of Hg(II) with EDTA. The absolute formation constant for Hg–EDTA has been determined as $K_f = 10^{22.1}$ M^{-1} (Casas & Jones, 1980), and at pH 7.4 the apparent binding constant is only slightly less, $10^{18.9}$ M^{-1}. An attempt to determine the activation energy for the $HgCl_2$ reduction by measuring the rate constant at temperatures higher than 10 °C was unsuccessful due to the rapidity of the reaction and will require fast reaction studies.

Mechanistically, electron transfers can be described as either inner-sphere or outer-sphere processes. Adopting the definitions of Saveant and co-workers (Lexa et al., 1988, 1990), in outer-sphere electron transfer reactions bond cleavage and formation either do not occur or occur separately from electron

[4] In the presence of excess DMPS, Hg(II) can form either a monodentate or bidentate complex:

$$DMPS + Hg(II) \rightarrow (DMPS)Hg + DMPS \rightarrow (DMPS)_2Hg$$

The absolute formation constants are large for both complexes: for Hg(DMPS) $K_f = 10^{42.2}$ M^{-1} and for $Hg(DMPS)_2$ $K_f = 10^{10.9}$ M^{-1} (Casas & Jones, 1980). However, at the pH used in these experiments (pH 4.7), the apparent formation constant for $Hg(DMPS)_2$ is substantially smaller ($K_f^{app} = 10^{-0.3}$ M^{-2}) than the absolute formation constant, and, given the low concentration DMPS, the Hg(II) is predominantly found as Hg(DMPS). Additionally, there is no evidence that Hg(II) is ligated by more than two sulfurs in $Hg(DMPS)_2$ (Basinger et al., 1981).

Table II: Dihydroflavin-Mediated Reduction of Metal Ions, Pseudo-First-Order Rate Constants for the Oxidation of 80 μM $FADH^-$ by Various Metal Ions (1.6 mM) in 100 mM HEPES, pH 7.4

metal ion	form[a]	temp (°C)	k' ($\times 10^{-3}$ min^{-1})	$E°$ (mV)[b]
Hg(II)	$HgCl_2$	10	1100	850
Hg(II)	Hg–EDTA	10	13	850
Au(II)	$AuBr_4^{1-}$	10	>4000	1290
Pt(II)	$PtCl_4^{2-}$	30	0	1200
Pd(II)	$PDCl_4^{2-}$	30	92	830
Ag(I)	Ag–EDTA	30	>4000	800
Fe(III)	Fe–EDTA	30	>4000	770
Cu(II)	Cu–EDTA	30	0	340
Pb(II)	Pb–EDTA	30	0	−120
Co(II)	$CoCl_2$	30	0	−280
Cd(II)	Cd–EDTA	30	0	−400

[a] Metal ion was dissolved in H_2O (1 M NaCl for $PdCl_2$) from the indicated salt or by complexation with EDTA (2.25 mM). [b] Formal potential for the uncomplexed metal ion, from Weast (1984).

transfer. In inner-sphere electron transfer reactions bond reorganization occurs simultaneously with electron transfer.

Previous studies have shown flavins to participate in outer-sphere electron transfers (Ahmad et al., 1981; Meyer et al., 1983). Mechanistic studies of one-electron transfers between metal oxidants and dihydroriboflavin (Singh et al., 1982b) and riboflavin semiquinone (Singh et al., 1982a) have shown that both are outer-sphere mechanisms. In accord with theoretical treatments of outer-sphere electron transfer processes (Marcus, 1964; Marcus & Sutin, 1985), the results presented in Table I demonstrate that the rate of reduction depends on the stability of the Hg(II)–ligand complex. This suggests that the dihydroflavin-mediated reduction of Hg(II) occurs via two sequential outer-sphere electron transfers. The failure to observe the semiquinone transiently is not unexpected since the flavin semiquinone readily disproportionates, generating its oxidized and fully reduced counterparts (Vaish & Tollin, 1971).

Reduction of Other Metallic Ions by $FADH^-$. One- and two-electron-reduced free flavins can reduce a variety of metal ions. Dihydroflavin, dihydroriboflavin (Distefano et al., 1989), and 3-(3-sulfopropyl)dihydrolumiflavin (Gopinath et al., 1989) undergo two-electron redox reactions with Hg(II) to generate Hg(0) and the oxidized flavin. The two-electron-reduced isoalloxazine moiety of a flavin-linked porphyrin can participate in a one-electron transfer reaction with Mn(III) porphyrin (Takeda et al., 1987). Similarly, dihydroriboflavin has also been shown to undergo one-electron transfer with both Co(III) and VO^{2+}, resulting in the stable (under reaction conditions of 1 M $HClO_4$) one-electron-reduced riboflavin semiquinone (Singh et al., 1982a). The riboflavin semiquinone itself can participate in one-electron transfers with a variety of metal ions, including Hg(II), Cu(II), Co(III), VO^{2+}, and Tl(III) (Singh et al., 1982b). Given the precedent of flavin–metal redox chemistry and the relevance of its to the MerA mechanism, the interaction of $FADH^-$ with various metal ions was examined.

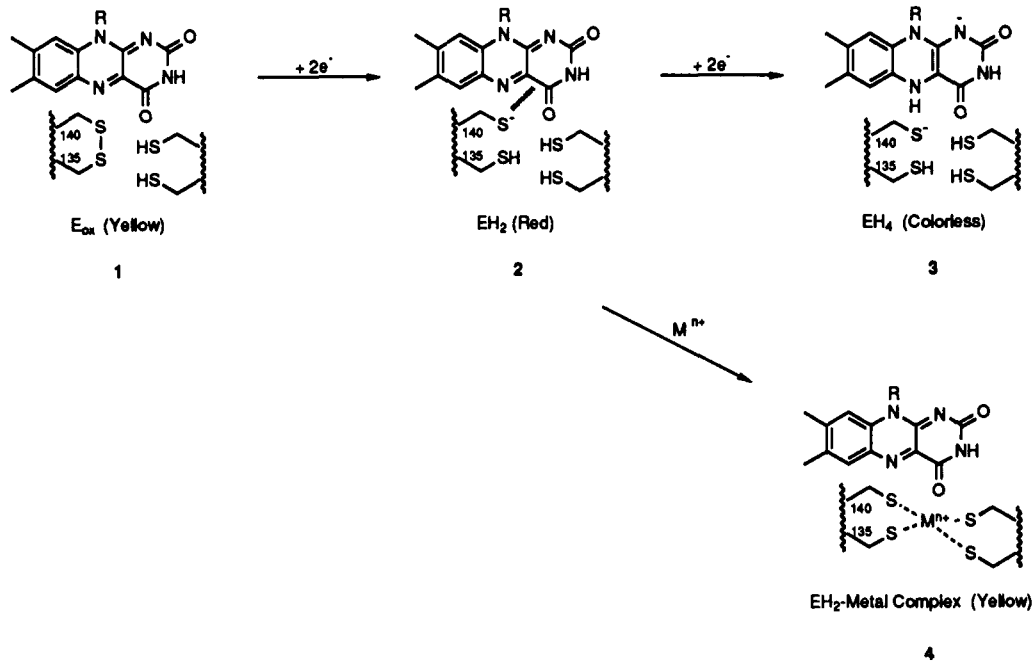
Single-turnover experiments were performed for the several other metal ions, and in four instances [Au(III), Ag(I), Pd(II), Fe(III)] dihydroflavin oxidation was observed. Pseudo-first-order rate constants were determined and are found in Table II. In all cases where redox chemistry occurred, the only product detected by UV–vis spectroscopy was the oxidized flavin, even in instances of one-electron reduction [e.g., Ag(I) → Ag(0)] where the semiquinone must have been generated. Presumably, any semiquinone which was generated rapidly underwent disproportionation. On the time scale of the experiments (minutes) the stable flavin semiquinone was not observed, as it has been for the dihydroriboflavin one-electron transfer reactions in the strongly acidic media 1 M $HClO_4$ (Singh et al., 1982a). Additionally, although flavin derivatives are known to form stable complexes with many metal ions (Hemmerich & Lauterwein, 1973), there was no evidence of complex formation between the metal ion and either dihydroflavin or the oxidized flavin.

The results shown in Table II indicate that $FADH^-$ can participate in redox reactions with several metal ions known to undergo one- and two-electron reductions. With the exception of Pd(II) and Hg(II) these reductions were quite rapid (>4.0 min^{-1}), and only a lower limit is reported. For the relatively slow Hg–EDTA and $PdCl_4^{2-}$ reductions, the reactions were determined to be biomolecular with second-order rate constants of 8.23 s^{-1} M^{-1} and 8.75 s^{-1} M^{-1} at 30 °C, respectively (data not shown). The $FADH^-$-mediated reduction of Au(III) was very rapid and presumably resulted in the formation of Au(I) since the Au(II) state is very unstable (Sadler, 1976). The resulting $AuBr_2^-$ complex is itself unstable, disproportionating to Au(III) and Au(0) (Sadler, 1976).

As shown in Table II, several ions did not undergo reduction. In these instances, the inability of $FADH^-$ to reduce the metal ions is probably a reflection of the unfavorable redox potential. The metal ion redox potentials have not been determined explicitly under the conditions used in the assay, and complexation by either solvent or EDTA will certainly lower them; however, an approximate correlation is seen between the formal potential for the metal ion and its ability to oxidize $FADH^-$. One apparent exception is $PtCl_4^{2-}$, which is not reduced by $FADH^-$. This is probably a reflection of the fact that Pt(II) complexes are typically more stable than the corresponding Pd(II) complexes (Cotton & Wilkinson, 1980). Additionally, Pt(II) may be forming aquo complexes or interacting with the HEPES buffer. At pH 7.4, the redox potentials of 3-ethyllumiflavin (Eberlein & Bruice, 1983) and MerA-bound FAD (Fox & Walsh, 1982) have been measured as −200 and −335 mV, respectively. Since the redox potentials of the unreactive metal ions in Table II are undoubtedly lower than this, $FADH^-$ oxidation is highly unfavorable.

Specificity of MerA in Metal Ion Reduction. The ability of free $FADH^-$ to reduce several metal ions in addition to Hg(II) prompted investigation of whether mercuric reductase will reduce other ions. The ability, or inability, of MerA to reduce alternate substrates should provide insight into the mechanism of Hg(II) reduction. As noted previously, MerA differs structurally from other disulfide oxidoreductases by the inclusion of a second dithiol pair in the active site. Beyond this, MerA is also found to diverge mechanistically from other family members (Miller et al., 1989). When isolated, all disulfide oxidoreductases contain an oxidized FAD and redox-active disulfide, a form denoted as E_{ox}. They also have available both two- and four-electron-reduced states, denoted the EH_2 and EH_4 forms, respectively. The three different forms are illustrated in Scheme II. With the possible exception of thioredoxin reductase, all disulfide oxidoreductases except mercuric reductase cycle between the E_{ox} and EH_2 states (Williams, 1991). In contrast, mercuric reductase cycles between the EH_2 and EH_4 states, with $FADH^-$ being required for turnover (Miller et al., 1989). The E_{ox}, EH_2, and EH_4 forms of MerA are spectrally distinct by UV–vis spectroscopy, and one can monitor their formation and decay readily. The spatial arrangement of the bound nicotinamide, the flavin, and the redox-active disulfide, as derived from the human gluta-

Scheme II



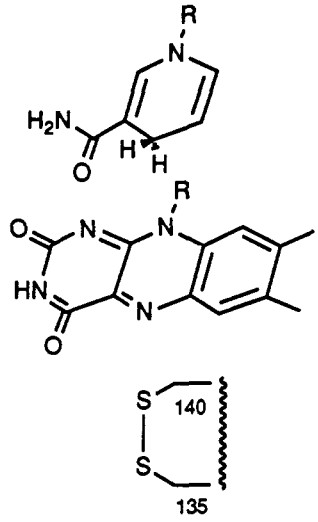


FIGURE 1: Spatial arrangement of the active site components of Tn501 MerA as derived from the X-ray crystal structures of *Bacillus* MerA (Schiering et al., 1991) and human GR (Pai & Schulz, 1983). Adapted from Moore et al. (1990).

thione reductase X-ray crystal structure (Pai & Schulz, 1983) and also the very recent determination of the *Bacillus* MerA structure (Scheiring et al., 1991), is depicted in Figure 1. Two-electron reduction of the E_{ox} form of MerA results in reduction of the redox-active disulfide, giving rise to a charge transfer between the oxidized flavin ring and the proximal thiolate (Cys_{140} in Tn501 MerA). The Cys_{140} thiolate is very close to the isoalloxazine ring [3.0 Å for the analogous $CysS_{212}^{-}$–C(4a) distance in *Bacillus* MerA (E. F. Pai, personal communication)], and its interaction with the ring lowers the pK_a substantially [pK_a = 5.2 for $Ser_{135}Cys_{140}$ MerA (Schultz et al., 1985)]. This interaction is manifested as a strong band in the absorbance spectrum (ϵ_{560nm} = 2050 m^{-1} cm^{-1}). In the partially reduced, catalytically incompetent EH_2 form of MerA (Scheme II, **2**), this charge transfer is at a maximum and solutions of the enzyme are red and not yellow as in E_{ox} (**1**). Reduction of EH_2 leads to the colorless, catalytically competent, EH_4 form (**3**), which has the necessary components for metal ion reduction: the reduced $FADH^-$ cofactor and an array of thiols to sequester the metal ion near the reductant. These spatial and electronic characteristics of the active site, coupled with the observation that free $FADH^-$ reduces a variety of metal ions (vide supra), suggested that the reduction of other metal ions was feasible.

Previously, Winkler and co-workers had examined the ability of *E. coli* R100 MerA to reduce other metal ions (Rinderle et al., 1983). Employing a multiple-turnover assay in which the MerA-mediated reduction was monitored by NADPH consumption, they found no metal ion, save Hg(II), was reduced. Since this assay is relatively insensitive, very slow or single-turnover reductions may have gone undetected. The situation is analogous to that seen for several MerA mutants in which the activity levels were too low to measure using this assay (Distefano et al., 1989). In these cases, the single-turnover assay developed by Williams was employed (Williams et al., 1979). In this assay, the enzyme is reduced to its active EH_4 form, and, under anaerobic conditons, a substrate is added. Subsequently, a single enzyme turnover event is monitored by the regain of oxidized flavin absorbance (A_{458}) since EH_2 MerA can bind Hg(II) (thereby resembling E_{ox}) but not reduce it (Miller et al., 1986). In the presence of excess substrate, a pseudo-first-order rate constant can then be determined. This assay is highly sensitive: control experiments suggest that activity levels of 1/100 000th wild-type MerA activity can be detected. In contrast, the background rate in the NADPH consumption assay under aerobic conditions is approximately 1000-fold higher.

Employing the single-turnover assay, the ability of wild-type MerA to reduce other metal ions was studied. Given the necessity of the four active site cysteines for bacterial Hg(II) detoxification, the ability of mutant MerAs to reduce alternative metals was not investigated. For these and subsequent experiments, MerA was digested with α-chymotrypsin to remove the first 85 amino acids. This "clipped" MerA is fully active in Hg(II) reduction, and any artifacts due to the $Cys_{10}Cys_{13}$ dithiol pair (e.g., competition for the metal ion) are removed. In a typical experiment, MerA was reduced with dithionite until the charge transfer band (λ_{560nm}) was approximately 20% of its maximal intensity. At this point the majority (80%) of the enzyme is in the fully active EH_4 form,

Table III: Pseudo-First-Order Rate Constants and Relative Rates of Hg(II) Reduction by Wild-Type MerA, Several Mutants, and $FADH^-$[a]

reductant	k' (min^{-1})	relative rate
CCCC (wild-type)[b]	340[c]	17000
ACCC	0.2	10
CACC	0.02	1
CCAC	5	250
CCCA	120	6000
CCAA	0.3	15
$FADH^-$	0.3	15

[a] Adapted from Moore et al. (1990). [b] See footnote 5. [c] The pseudo-first-order rate constant given here differs from that in Table I. This is due to the biphasic nature of the reduction which consists of an initial fast phase (780 min^{-1}) and a slower second phase (340 min^{-1}). See Fox and Walsh (1982).

and there remains no excess dithionite which could also reduce the metal ion. To the prereduced enzyme was added an excess of the metal ion, and the reaction was monitored for the formation of FAD (A_{458nm}) for a period of 0.5–2 h. Several ions were tested, including those which were reduced by free $FADH^-$ [Pd(II), Au(III), Ag(I), Fe(III)] and several that were not [Pt(II), Pb(II), Cu(II), Co(II), Cd(II)]. MerA shows exclusivity for Hg(II): none of the other metal ions were reduced by the enzyme. Nevertheless, the reduced enzyme was fully active, and addition of $HgCl_2$ to the inactive EH_4–metal ion mixtures gave, in all instances, very rapid formation of FAD with the oxidation reaching completion in less than 10 s.

The inability of other metal ions to inhibit Hg(II) reduction in the single-turnover assay could have arisen from their failure to occupy the active site. Evidence against this was the observation by Winkler and co-workers that, in their multiple-turnover assay, several metal ions functioned as either competitive [Cd(II), Ag(I), Cu(II)] or noncompetitive [Au(III)] inhibitors of *E. coli* R100 MerA (Rinderle et al., 1983). These results indicated that the metal ions bound at, or near, the active site. However, in the single-turnover assay, MerA failed to reduce the other metal ions, and Hg(II) reduction was not inhibited by them. These observations are consistent with the other metal ions not occupying the active site. Given the disparity between these two sets of observations, an assay was developed to determine whether the metal ions were binding at the active site.

Binding of Other Metals to the MerA Active Site. To monitor active site metal ion binding, the interaction of EH_2 MerA with various metal ions was investigated. As shown in Scheme II, binding of a metal ion to the Cys_{140} thiolate of EH_2 MerA removes the charge transfer interaction (Scheme II, **2** → **4**). Therefore, the spectral characteristics of EH_2 MerA which has a metal ion bound at Cys_{140} will resemble E_{ox}. Under these conditions, binding of metal ions to Cys_{140} is easily monitored by observing the loss of the charge transfer band.

Several lines of evidence have suggested that Cys_{140} is the most critical of all four active site cysteines. Single mutations of this residue to either serine (CSCC) or alanine (CACC)[5] have resulted in mutant enzymes with only 1/8000th and 1/17 000th wild-type activity, respectively (Distefano et al., 1989). As shown in Table III, of all the single Cys → Ala mutations studied, the protein CACC was the one most strongly compromised in enzymatic activity. The proximity of Cys_{140} to the flavin also suggests its importance: Cys_{140} is the residue closest to $FADH^-$, the discrete reductant in MerA catalysis (Distefano et al., 1989). Other disulfide oxidoreductases are believed to utilize the charge transfer thiolate–C4a covalent adduct in redox-active disulfide reduction (Williams, 1991), and it has been demonstrated that the MerA triple mutant ACAA can form a $Cys_{140}S$–C4a adduct (Miller et al., 1990). The accumulated evidence suggests that binding to Cys_{140} is necessary for reduction.

Table IV: Quenching of FAD–Cys_{140} Charge Transfer by Various Metal Ions

metal ion	% quenching of CT band[a]	metal ion	% quenching of CT band[a]
Hg(II)	83.6	Cd(II)	47.3
Ag(I)	95.4	Cd(II)	39.6 (pH 8.0)[b]
$CH_3Hg(I)$	57.2	Cd(II)	32.5 (pH 7.4)[b]
Au(III)	34.9	control	16.0
Pd(II)	41.9		

[a] Decrease in A_{560} after addition of 1 equiv of metal ion. [b] 100 mM HEPES.

For the most part, metal ion binding experiments were carried out in 100 mM CHES, pH 9.0, as this buffer system was found to be good for achieving comparatively high levels of binding. Similar metal ion binding trends were observed at lower pH's, but the quenching intensity was attenuated, reflecting the importance of the other thiols in the binding of the metallic species. At pH 9.0, a significantly greater portion of the active site cysteine residues are found as thiolates and can participate in binding. Unlike Cys_{140}, the other three cysteine thiolate side chains are not strongly stabilized and have pK_a's closer to that of glutathione [pk_{SH} = 8.81 (Cheesman et al., 1988)] (Miller and Cummings, unpublished results). The enzyme remains viable in 100 mM CHES, pH 9.0, as judged by the aerobic NADPH consumption assay with MerA retaining 70% of its activity in this buffer as compared to its usual assay buffer 80 mM potassium phosphate, pH 7.4 (Fox & Walsh, 1982).

Studies on metal ion binding to the Cys_{140} thiolate were carried out by monitoring the loss in charge transfer absorbance of the EH_2 form. Under anaerobic conditions, MerA (30 μM) was reduced with dithionite past the point of maximal charge transfer and until this band was 80% of its maximum.[6] This mixture of EH_2 and EH_4 was then titrated by addition of the metal ion in 0.5 equiv increments, and the decrease in A_{560} was monitored. A total of 5 equiv was added (10 data points). As a control, the addition of H_2O without metal ion was also performed. The results from titration of the MerA charge transfer band with several metal ions are given in Table IV.

As assessed by quenching of the charge transfer band, several metal ions showed a high affinity for the Cys_{140} thiolate. The control indicated that the assay had a relatively high background (8% quenching per addition) with a smooth monotonic decrease in A_{560} nm (R = 0.994, N = 10). With this background limitation of signal to noise, no detectable binding of Fe(III), Co(II), and Pt(II) was observed. For those

[5] A shorthand notation has been adopted for describing MerA mutants in which one of the four active sites cysteines has been altered. A four-letter code is utilized in which each letter denotes one of the four active site cysteine positions; thus, wild-type ($Cys_{135}Cys_{140}Cys_{558}Cys_{559}$) is CCCC, and the Cys_{140} → Ala_{140} mutant is CACC.

[6] By reducing the enzyme past the point of maximal charge transfer (i.e., to an EH_2/EH_4 mixture), differentiation between metal reduction and metal binding was obtained. At maximal charge transfer the majority of the enzyme is in the EH_2 form and *both* metal binding and metal reduction result in an increase in A_{458} and a decrease in A_{560}. In contrast, for the EH_2/EH_4 mixture, metal reduction results in an increase in both A_{458} and A_{560} (as EH_4 → EH_2) whereas metal binding results in an increase in A_{458} and a decrease in A_{560}.

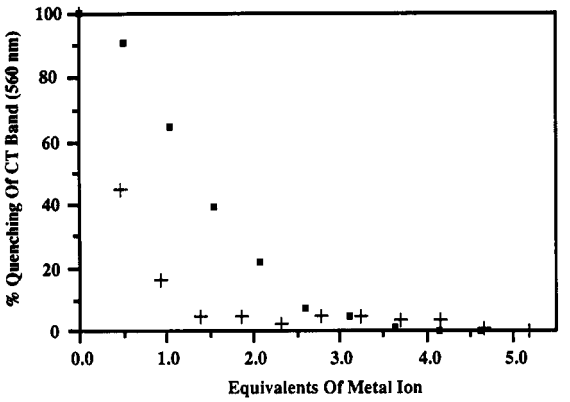


FIGURE 2: Quenching of charge transfer of Cys_{140} by titration with $KAuBr_4$ (···) or $HgCl_2$ (+++) in 100 mM CHES, pH 9.0 ([MerA] = 30 μM).

ions reported in Table IV, smooth titration curves were obtained. As representative examples, the titrations of EH_2 MerA with $HgCl_2$ and $AuBr_4^{1-}$ are presented in Figure 2. There is a correlation between preferred coordination number and active site affinity (Cotton & Wilkinson, 1980). Ions which prefer bis-coordination and have a high affinity for thiolates such as Hg(II) and Ag(I) bind most strongly to Cys_{140}. Indeed the organomercurial $CH_3Hg(I)$, although comparatively large, has a moderate affinity for the thiolate. In contrast, ions such as Cd(II) and Au(III) prefer to form four-coordinate complexes and bind less tightly. Metal ions which often are found as higher coordinate species, such as octahedral Fe(III) and Co(II) complexes, bind very poorly if at all.

In principle, the formation constant for Hg(II) in the MerA active site could be determined by observing the amount of Hg(II)-induced quenching of EH_2 MerA in the presence of other thiols with known formation constants. The value obtained could then be compared to that calculated from the K_m data ($K_f^{app} = 10^{39.8}$ M^{-1} for Tn501 MerA). Attempts to carry out this experiment were unsuccessful. It was found that exogenous thiols could readily reduce the flavin, thereby converting EH_2 MerA to EH_4 MerA and leading to the reduction of the bound Hg(II).

It was assumed that the binding affinity of the charge transfer EH_2 form and the catalytically competent EH_4 form were similar, that once binding to Cys_{140} occurred the EH_2 form took on spectral characteristics similar to E_{ox}, and that reduction was not occurring with the redox labile metals. To demonstrate that this model was correct, the binding of Au(III) to MerA was studied further (Figures 3 and 4). In Figure 3, several species from a titration of MerA with dithionite and Au(III) are depicted. The initial spectrum is that of E_{ox} with its λ_{max} at 458 nm and shoulder at 480 nm. Reduction of E_{ox} gives first the EH_2 form (λ_{max} = 438 nm, CT band centered at 540 nm) and, upon further reduction, colorless EH_4 MerA. Also depicted in Figure 3 is an EH_2/EH_4 mixture in a ratio of 76:24 as calculated by the decrease in A_{560}. To this mixture was added $AuBr_4^{1-}$ such that approximately half of the CT band was quenched. The resulting spectrum is also presented in Figure 3. This spectrum clearly shows E_{ox} character with a maximum at 452 nm and a shoulder at 476 nm. To more rigorously show that EH_2 with metal bound resembled E_{ox}, a theoretical curve was constructed and compared to the actual result. Given that addition of Au(III) quenched the charge transfer 53.2%, the resulting spectrum should resemble a composite of E_{ox} and EH_2 which reflects the 53.2:46.8 ratio. Such a composite was constructed by computer fitting and compared to the actual result as shown in Figure 4. The

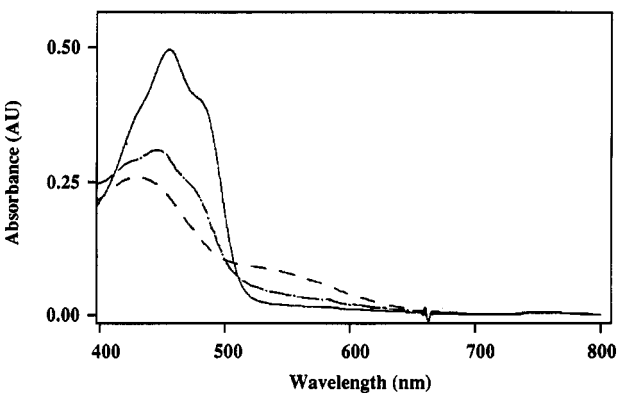


FIGURE 3: Titration of MerA (50 μM) with $KAuBr_4$ in 10 mM CHES, pH 9.0. Spectra are as follows: E_{ox} (—), EH_2/EH_4 mixture prior to addition of Au(III) (– –), and EH_2/EH_4 mixture after addition of Au(III) (–·–).

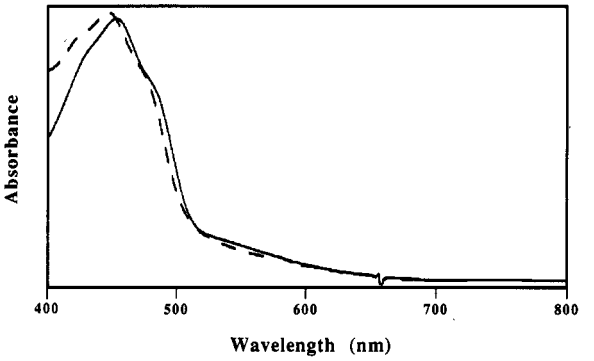


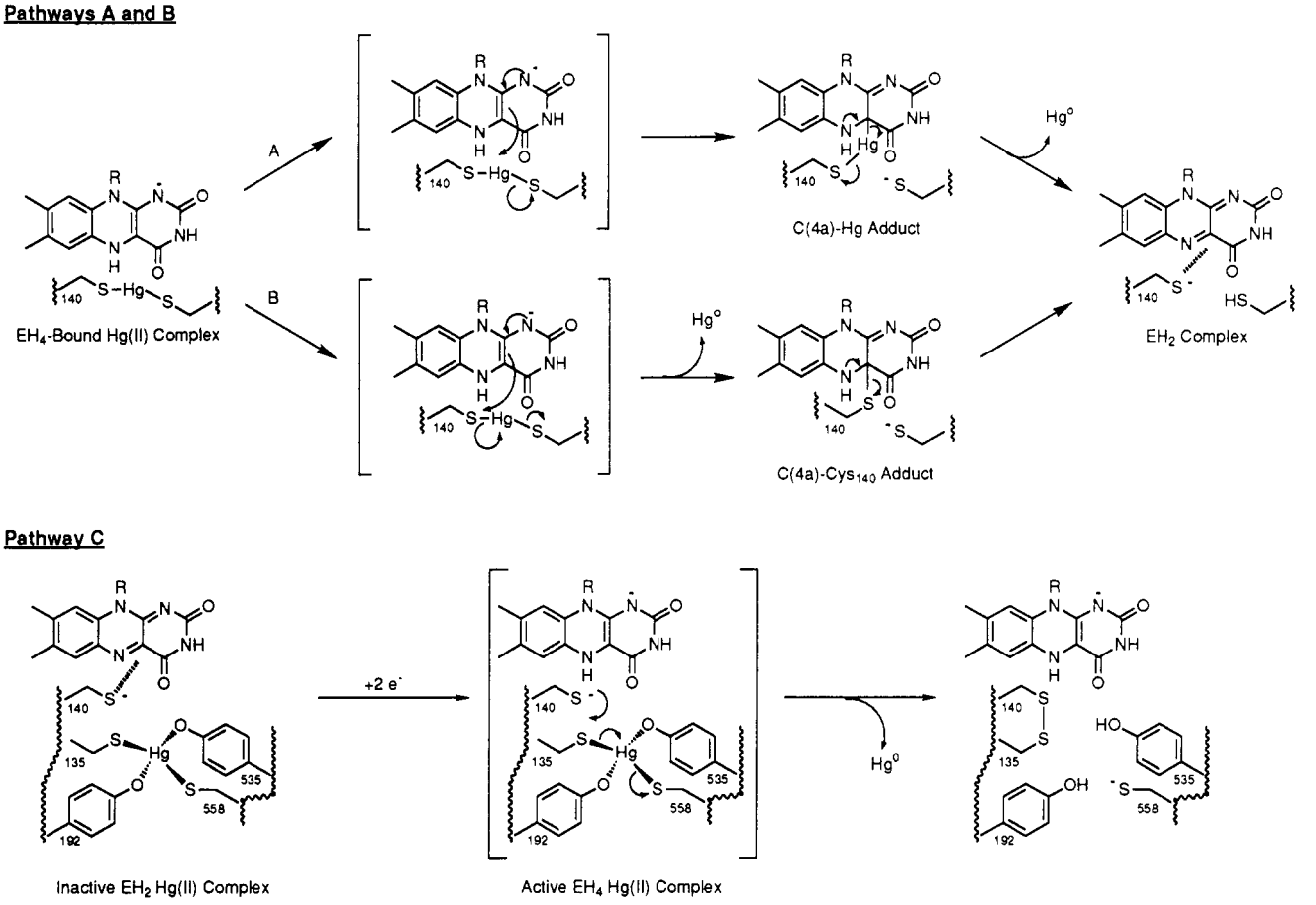
FIGURE 4: Comparison of theoretical (—) and actual (– –) MerA–Au(III) complexes.

agreement is striking and demonstrates that the EH_2 form assumes spectral characteristics similar to E_{ox} upon metal binding. Additionally, the relative absorbances of the spectra are nearly equal, indicating that binding of Au(III) to MerA does not perturb the EH_2 to EH_4 ratio and, hence, no reduction is occurring. Similar results were obtained with other metal ions. That $AuBr_4^{1-}$ is no longer present is also shown by the close agreement between the theoretical and actual spectra since $AuBr_4^{1-}$ absorbs strongly in the visible region of the spectrum (ϵ_{450nm} = 1500 M^{-1} cm^{-1}).

The results given in Table IV compare well to those of previous workers (Rinderle et al., 1983). For those ions which inhibited MerA activity and were also tested in the Cys_{140} binding assay [Ag(I), Cd(II), Au(III), Fe(III), and Co(II)], an identical ordering was found. This is consistent with the observed inhibition of *E. coli* R100 MerA by various metal ions being due to their binding to the charge transfer thiolate. The inability of other metal ions to inhibit Hg(II) reduction in the single-turnover assay was most likely due to the absence of β-mercaptoethanol in the buffer. Without a competing thiol such as β-mercaptoethanol, Hg(II) (introduced as $HgCl_2$) is much more likely to partition into the active site and rapidly be reduced. The exchange rates of thiolates with Hg(II) are sufficiently fast as to have gone undetected in the single-turnover assay (Cheesman et al., 1988).

The report that Au(III) functions as a noncompetitive inhibitor of R100 MerA in the multiple-turnover assay is at variance with the data presented here (Rinderle et al., 1983). One explanation is that there may be subtle differences in the active sites of Tn501 MerA and R100 MerA such that Au(III) is not bound in an identical manner in the two enzymes. Additionally, under the assay conditions reported by Rinderle and co-workers, it is likely that the Au(III) had undergone

Scheme III



spontaneous reduction to Au(I), a metal ion which has a different coordination sphere and was not tested in the active site binding assay. Either of these conditions (or an alternate one) could lead to the observed disparity.

A Possible Mechanism for Selective Reduction of Mercuric Ion. Although free $FADH^-$ reduces Au(III), Ag(I), and Pd(II) at rates comparable to that of Hg(II), MerA reduces none of them in single-turnover assays. However, the charge transfer quenching by them confirms their substantial affinities for the active site and failure to undergo reduction is not due to lack of access. Clearly active site binding is a necessary but insufficient requisite for enzymatic $FADH^-$-mediated reduction.

Hg(II), Ag(I), Pt(II), and Au(III) ions are all highly redox labile and have high affinities for the MerA active site, yet the enzyme-mediated redox outcome of their binding is strikingly different. The rate of enzymatic Au(III), Ag(I), or Pt(II) reduction, if it occurs at all, is less than 1/100 000th that of Hb(II). What can this apparent paradox suggest about the mechanism of enzymatic Hg(II) reduction? In principle, $FADH^-$ could reduce bound Hg(II) by either an inner-sphere or outer-sphere electron transfer mechanism, and the results found for free dihydroflavins and Hg(II) in the presence of different ligands (Table I) are in accord with an outer-sphere electron transfer mechanism. Additionally, the participation of flavins in outer-sphere electron transfer reactions is well documented (Ghisla & Massey, 1989; Meyer et al., 1986; Thorpe, 1991). Reaction rates for outer-sphere mechanisms depend critically on the overall redox potential and the distance between the redox partners (Gray & Malmstrom, 1989; Meyer et al., 1983). The failure of metal ions other than Hg(II) to undergo reduction in the MerA active site is not obviously distance related. Could it be due to an unfavorable redox potential? Although the redox potentials of the metal ions have not been measured explicitly under the reaction conditions, the formal potentials of Au(III) and Ag(I) with thiocyanato ligands have been reported: for the one-electron reduction of AgSCN it is 90 mV (Vanysek, 1984), and for the two-electron reduction of $Au(SCN)_4^-$ it is 615 mV (Sadler, 1976), both well above redox potential of −335 mV measured for $FADH^-$ in MerA (Distefano et al., 1989). Further, the stabilization afforded to these ions by thiol ligation is similar in magnitude to that afforded to Hg(II), suggesting that mercuric ion reduction should be equally compromised, which it is not. These results suggest that the mechanism of MerA-mediated reduction of Hg(II) involves unique chemistry and/or ligation of Hg(II).

Failure to observe reaction for other coordinated metal ions is consistent with MerA-mediated reduction of Hg(II) utilizing an inner-sphere electron transfer mechanism rather than an outer-sphere mechanism. Inner-sphere mechanisms are much more sensitive to spatial constraints than outer-sphere reactions, often having significantly greater entropies of activation and, hence, higher selectivity (Lexa et al., 1990). The inability of other metal ions to undergo MerA-mediated reduction may result from their assuming a distorted, or alternate, coordination. These coordination differences would have a pronounced effect on an inner-sphere reaction since it depends on the spatial relationship of the reacting partners. Under the constraints imposed by an inner-sphere mechanism, the exclusivity of MerA would derive from the specific tailoring of the active site to bind only Hg(II) in a reactive conformation. Other metal ions either do not adopt or significantly distort this conformation, precluding further reaction.

Inner-sphere reactions are less well established than outer-sphere reactions for reduced flavins; one notable exception

is the work of Bruice and co-workers who demonstrated that N5-alkylated dihydroflavin derivatives can react with O_2 to form 4a-hydroperoxyflavins (Kemal et al., 1977). Also, disulfide oxidoreductases can form C(4a) FAD adducts: this species has been detected in monoalkylated lipoamide dehydrogenase (Thorpe & Williams, 1981), EH_2 thioredoxin reductase containing 1-deazaFAD (O'Donnell & Williams, 1984), the MerA mutant ACAA (Miller et al., 1990), and (transiently) wild-type MerA at pH 5.1 (Sahlman et al., 1986). Additionally, MerA catalyzes the O_2-dependent oxidation of NADPH to $NADP^+$, a reaction thought to proceed through a 4a-hydroperoxyFAD intermediate (Distefano et al., 1989).

The close proximity of the Cys_{140} to the C(4a) position of FAD, its strong thiolate character at physiological pH, and its ability to form a C(4a) adduct in the ACAA mutant are consistent with a mechanistic pathway involving a C(4a) adduct. Two pathways involving such an intermediate are illustrated in Scheme III. The starting point for both pathways is the EH_4–Hg(II) complex, which is ready to undergo reduction. In this complex, Hg(II) is ligated by Cys_{140} and one (or more) additional cysteine residue(s). Pathway A involves initial attack of the $FADH^-$ on the Hg(II) ion with displacement of a thiolate, giving the C(4a)–Hg covalent adduct. This intermediate would break down by loss of the N5 proton and reductive elimination of Hg(0). In pathway B $FADH^-$ initially attacks sulfur with the concomitant loss of Hg(0). As in pathway A, the CysS–C(4a) adduct thus generated breaks down by reductive elimination. At this time the extent to which either (or both) of these pathways is operative cannot be distinguished although the failure of MerA to reduce Au(III) and Pd(II) seems more in line with the formation of a C(4a)–Hg intermediate than a CysS–C(4a) adduct.

A third, and rather divergent, mechanism is also presented in Scheme III (pathway C). This mechanism takes into account the X-ray crystallographic results recently obtained for *Bacillus* sp. strain RC607 (Scheiring et al., 1991). The crystal structure of this enzyme revealed not only that the second dithiol pair is in the active site but also that two tyrosines are part of the active site. These tyrosines are also conserved in all other mercuric reductases sequenced to date, including Tn501 MerA (Tyr_{192} and Tyr_{535}). These tyrosines, along with Cys_{207} and Cys_{628} (analogous to Cys_{135} and Cys_{558} in Tn501 MerA), were found to bind Cd(II) in a distorted tetrahedral complex. The inner-sphere mechanism presented here does not utilize $FADH^-$ as the discrete reductant, nor is Cys_{140} bound to the Hg(II) ion. Instead, Hg(II) is bound in the distorted tetrahedral complex observed for Cd(II) in *Bacillus* MerA. Reduction of FAD nullifies the charge transfer interaction with Cys_{140}, thereby increasing the nucleophilicity of the thiolate. The more reactive Cys_{140} then reforms the redox-active disulfide with concomitant expulsion of Hg(0). The resulting enzyme species is in equilibrium with EH_2 MerA and readily converts to it, regenerating the Hg(II)-binding site. This mechanism is analogous to that of human glutathione reductase in which the proposed rate-determining step is deprotonation of the imidazolium ion of His_{467} (Wong et al., 1988). Once this occurs, the charge transfer thiolate is more reactive and forms the redox-active disulfide, resulting in the displacement of the second glutathione molecule. In the *Bacillus* MerA structure, His_{467} has been replaced by Tyr_{605}, and, if destabilization of the thiolate is necessary, it must be achieved in another manner. One drawback to this mechanism is that Hg(II) is not bound to Cys_{140} in EH_2 MerA, in disagreement with results presented previously (Miller et al., 1986) and in this report. However, it may be that the EH_2–Hg(II) complex presented in pathway C is not the most stable species (which the binding assay would have detected) but, rather, a destabilized intermediate on the reaction pathway.

The results presented in this paper are most consistent with MerA utilizing an as yet undefined inner-sphere electron transfer to reduce Hg(II). This is in contrast to other reduced flavin–metallic ion redox reactions, all of which utilize an outer-sphere electron transfer mechanism. These data show that the spatial and/or electronic characteristics of the active site are well defined with the specificity of mercuric reductase for Hg(II) reduction probably arising from either the formation of covalent bonds or ligation spheres which are unique for Hg(II). Recently, evidence that the active sites in the MerA homodimer are functionally differentiated has been presented (Miller et al., 1991). It was suggested that this "half-sites" asymmetry may serve a role in catalysis by allowing the formation of transiently destabilized Hg(II) active site complexes. The specificity of MerA may reflect its inability to achieve these destabilized species with other metallic ions. In regard to Hg(II) reduction, the specific roles of the individual residues within the active site and the identification of intermediates on the reaction pathway will become more clear with additional mutagenesis and the application of methods such as X-ray diffraction, NMR, and stopped-flow experiments to wild-type and mutant mercuric reductases.

Registry No. FAD, 146-14-5; Cl^-, 16887-00-6; EDTA, 60-00-4; $FADH^-$, 1910-41-4; Hg, 7439-97-6; Au, 7440-57-5; Pt, 7440-06-4; Pd, 7440-05-3; Ag, 7440-22-4; Fe, 7439-89-6; Cu, 7440-50-8; Pb, 7439-92-1; Co, 7440-48-4; Cd, 7440-43-9; Cys, 52-90-4; MerA, 67880-93-7.

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