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Evidence for the Participation of Cys_{558} and Cys_{559} at the Active Site of Mercuric Reductase†

Susan M. Miller,*,‡ Melissa J. Moore,§ Vincent Massey,‡ Charles H. Williams, Jr.,‡,∥ Mark D. Distefano,§ David P. Ballou,‡ and Christopher T. Walsh§

Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, and Veterans Administration Medical Center, Ann Arbor, Michigan 48105

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ABSTRACT: Mercuric reductase, with FAD and a reducible disulfide at the active site, catalyzes the two-electron reduction of Hg(II) by NADPH. Addition of reducing equivalents rapidly produces a spectrally distinct EH_2 form of the enzyme containing oxidized FAD and reduced active site thiols. Formation of EH_2 has previously been reported to require only 2 electrons for reduction of the active site disulfide. We present results of anaerobic titrations of mercuric reductase with NADPH and dithionite showing that the equilibrium conversion of oxidized enzyme to EH_2 actually requires 2 equiv of reducing agent or 4 electrons. Kinetic studies conducted both at 4 °C and at 25 °C indicate that reduction of the active site occurs rapidly, as previously reported [Sahlman, L., & Lindskog, S. (1983) *Biochem. Biophys. Res. Commun. 117*, 231-237]; this is followed by a slower reduction of another redox group via reaction with the active site. Thiol titrations of denatured E_{ox} and EH_2 enzyme forms show that an additional disulfide is the group in communication with the active site. [^{14}C]Iodoacetamide labeling experiments demonstrate that the C-terminal residues, Cys_{558} and Cys_{559}, are involved in this disulfide. The fluorescence, but not the absorbance, of the enzyme-bound FAD was found to be highly dependent on the redox state of the C-terminal thiols. Thus, E_{ox} with Cys_{558} and Cys_{559} as thiols exhibits less than 50% of the fluorescence of E_{ox} where these residues are present as a disulfide, indicating that the thiols remain intimately associated with the active site. Initial velocity measurements show that the auxiliary disulfide must be reduced before catalytic Hg(II) reduction can occur, consistent with the report of a preactivation phenomenon with NADPH or cysteine [Sandstrom, A., & Lindskog, S. (1987) *Eur. J. Biochem. 164*, 243-249]. A modified minimal catalytic mechanism is proposed as well as several chemical mechanisms for the Hg(II) reduction step.

Mercuric reductase (MR)[1] plays a crucial role in bacterial detoxification of mercurials as it catalyzes the $2e^-$ reduction of Hg(II) shown in eq 1. The enzyme exhibits extensive similarities to the pyridine nucleotide disulfide oxidoreductases, lipoamide dehydrogenase and glutathione reductase, both in primary sequence and in spectral properties. Thus, they all

$$Hg(SR)_2 + NADPH + H^+ \rightarrow Hg^0 + NADP^+ + 2RSH \quad (1)$$

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* To whom correspondence should be addressed.
‡ University of Michigan.
§ Harvard Medical School.
∥ Veterans Administration Medical Center.

[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; E_{act}, mercuric reductase containing oxidized FAD and oxidized active site thiols, but reduced C-terminal thiols; EH_2, mercuric reductase containing oxidized FAD and the active site thiols reduced; EH_4, mercuric reductase containing $FADH_2$ and reduced active site thiols; E_{ox}, mercuric reductase containing oxidized FAD and active site disulfide; HPLC, high-performance liquid chromatography; MR, mercuric reductase; 4-PDS, 4,4′-dithiodipyridine; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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contain FAD and a redox-active disulfide ($Cys_{135}Cys_{140}$ in MR) in close proximity at the active site (Williams, 1976; Fox & Walsh, 1982). By analogy to lipoamide dehydrogenase and glutathione reductase (Massey & Ghisla, 1974), partial reduction of mercuric reductase affords an EH_2 species whose spectral features have been attributed to a charge-transfer interaction between the thiolate of Cys_{140} and FAD (Fox & Walsh, 1982). Furthermore, both aerobic low-temperature rapid reaction studies (Sahlman et al., 1984) and aerobic thiol titrations of denatured E_{ox} and EH_2 enzyme forms (Fox & Walsh, 1982) indicate that formation of EH_2 requires only 2 electrons and results in reduction of only the active site disulfide. These results are exactly as were seen with lipoamide dehydrogenase and glutathione reductase (Massey et al., 1960; Massey & Williams, 1965; Williams, 1976) and suggest that mercuric reductase and the pyridine nucleotide disulfide oxidoreductases are quite similar in their active site structures. However, mercuric reductase readily catalyzes the reduction of Hg(II) while lipoamide dehydrogenase and glutathione reductase cannot reduce Hg(II) at a useful rate [see Moore and Walsh (1989)] and are severely inhibited toward their normal disulfide interchange reactions by binding of mercurials at the active site dithiol of reduced enzyme (Massey & Williams, 1965; Casola & Massey, 1966). Thus, there must be some additional structural elements involved in Hg(II) binding in mercuric reductase that prevent formation of the same type of inhibited complex.

We show here several lines of evidence that the C-terminal cysteine thiol pair, $Cys_{558}Cys_{559}$, forms an integral part of the active site of mercuric reductase. First, these thiols form a disulfide upon enzyme isolation that is reduced by NADPH via thiol/disulfide interchange with the active site thiol pair. Second, the enzyme flavin fluorescence is dramatically affected by the redox state of these cysteines, indicating their proximity to the flavin. Finally, the disulfide form of the enzyme shows no initial activity in catalytic assays, whereas the dithiol form shows normal activity. Furthermore, preincubation of the disulfide form with NADPH or cysteine gives activation as reported by Sandstrom and Lindskog (1987). A catalytic mechanism incorporating these results is presented. It should be noted that intramolecular dithiol/disulfide interchange has also been proposed in the B1 subunit of *Escherichia coli* ribonucleoside diphosphate reductase (Lin et al., 1987).

Experimental Procedures

Materials

Wild-type mercuric reductase was obtained from *E. coli* W3110 $lacI^q$ containing the plasmid pPS01 [which contains a subclone of the Tn*501* mercuric reductase (*merA*) gene]. The $Ala_{10}Ala_{13}$ mutant mercuric reductase was obtained from the same strain containing the plasmid pMMOa10a13 described in the preceding paper (Moore & Walsh, 1989). Purification of both enzymes was as previously described for the wild type (Schultz et al., 1985), except that (1) no protease inhibitors were used since they appear to be ineffective in preventing proteolysis with this enzyme and (2) 1 mM $NADP^+$ was used in place of 50 μM NADPH to elute the enzyme from the Orange A column in order to avoid production of H_2O_2 due to turnover with O_2 during purification. After the Orange A column, the enzyme was frozen quickly (dry ice/acetone) and stored at −70 °C in the Orange A elution buffer (20 mM sodium phosphate, pH 7.3, 0.5 mM EDTA, 0.1% 2-mercaptoethanol, 1 mM $NADP^+$) + 10% (w/v) glycerol. Before use, enzyme was separated from tightly bound $NADP^+$ by addition of 2 M KBr followed by five successive concentration/dilution cycles using 50 mM potassium phosphate/0.3 mM EDTA buffer, pH 7.3, in a Centricon 30 microconcentrator. It was then precipitated with ammonium sulfate (75%) in the presence of 100 μM FAD and, finally, desalted by gel filtration on G-25 in 50 mM potassium phosphate/0.3 mM EDTA buffer, pH 7.3 (then stored in this form at 0–4 °C). All studies were made with this KBr-treated enzyme. All chemicals were of the highest grade available and were used without further purification.

Methods

All experiments were conducted in 50 mM potassium phosphate, pH 7.3, unless otherwise indicated. UV–vis spectra were recorded with Cary 17 or 219 double-beam spectrophotometers or a Hewlett-Packard 8452A diode array spectrophotometer. Fluorescence spectra were recorded with a scanning ratio spectrofluorometer built by Gordon Ford and Dr. David Ballou at the University of Michigan. Rapid reaction studies were carried out with a stopped-flow spectrophotometer with scanning capabilities (2-cm optical path) as previously described (Beaty & Ballou, 1981). Anaerobic titrations were conducted in cuvettes with two side arms similar to those previously described (Williams et al., 1979). Samples for anaerobic titrations contained 200 μM protocatechuate and were treated with 0.1–0.2 unit of protocatechuate dioxygenase [*Pseudomonas cepacia* (originally named *P. putida*); Bull & Ballou, 1981] following several evacuation/nitrogen equilibration cycles. Nitrogen gas was made anaerobic by passing over a heated column of Ridox (Fisher).

Thiol Titrations. Typically, in an anaerobic cuvette under N_2, 0.6 mL of ca. 7 μM mercuric reductase in 50 mM potassium phosphate, pH 7.3, was denatured by addition of 23 μL of 0.3 M EDTA and 0.7 mL of 8 M guanidine hydrochloride/20 mM Tris with an apparent pH of 8.03 (the resulting pH was 6.2 due to dissociation of guanidine hydrochloride upon dilution). Spectra were recorded before and after denaturation to accurately measure enzyme concentration. To this mixture was added 50 μL of a 10 mM ethanolic solution of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (ca. 100-fold excess over enzyme monomer), and the ΔA_{412} was measured [ΔE_{412} = 13.6 mM^{-1} (Beutler et al., 1963)]. For larger volumes of enzyme samples, proportionately larger volumes of the other reagents were used.

EH_2–NADPH complexes of the various enzymes were formed by anaerobic addition of 4–10 equiv of NADPH, T = 25 °C. When no further absorbance changes occurred at 340 nm, the thiol titer was determined anaerobically as described above.

DTT-treated E_{ox} enzymes were prepared by incubation of the enzyme with 2 mM dithiothreitol at 25 °C for 20 min followed by precipitation with ammonium sulfate (75% saturation, T = 0–4 °C) in the presence of 100 μM FAD. The sample was freed of excess reagents by filtration on Sephadex G-25 in 50 mM potassium phosphate/0.3 mM EDTA, pH 7.3. This procedure resulted in oxidation of the active site dithiol pair ($Cys_{135}Cys_{140}$) and the FAD, but not the other thiols in the enzyme (see Results).

Enzyme Assays. All assays were conducted at 25 °C in 50 mM potassium phosphate buffer, pH 7.3, containing 50 μM NADPH, 1 mM 2-mercaptoethanol, 50 μM $HgCl_2$, and 10 nM enzyme monomer.

[^{14}C]*Iodoacetamide Labeling Studies.* (*A*) *Identification of Cysteine-Containing Peptides.* Enzyme samples (ca. 20 nmol in 0.5 mL of 50 mM sodium phosphate, pH 7.5) were denatured by adding 480 mg of urea and then adjusting the volume to 1.0 mL with buffer (final urea concentration = 8

M). Enzyme disulfides were reduced by DTT (100 μM) followed by incubation at 37 °C for 1 h. Exhaustive alkylation was initiated by addition of [^{14}C]iodoacetamide (Amersham, 2.65 mCi/mmol) to a final concentration of 1 mM. After incubation for 1 h at 25 °C, the alkylation reaction was stopped by addition of 10 μL of 2-mercaptoethanol. Excess reagents were removed by dialysis against 100 volumes of 8 M urea, followed by dialysis against three changes of 100 volumes of 2 M urea dissolved in 100 mM Tris-HCl, pH 8.0. Subunit labeling stoichiometry was determined by comparing the radioactivity and protein content of each sample. The radioactivity in 50-μL aliquots of each sample dissolved in National Diagnostics Liquiscent was measured by using a Beckman LS-100 liquid scintillation counter. Protein was determined by using the Bio-Rad (Bradford) protein assay with wild-type mercuric reductase as the standard. Proteolysis was carried out by digesting 250 μL of each sample with TPCK-treated trypsin (Worthington, 20 μg/mL) for 6 h at 25 °C. Digestions were stopped by addition of 25 μL of glacial acetic acid, and samples were stored at 4 °C. Peptides were separated by reverse-phase chromatography on a Waters Associates HPLC equipped with an analytical wide-bore Vydac Protein and Peptides C-18 column. A linear gradient from 5% to 35% acetonitrile in aqueous 0.1% trifluoroacetic acid was run at 1.0 mL/min for 60 min. Fractions (1 mL) were collected and counted as above. Peptide labeling stoichiometry was determined by multiplying the percentage of the total counts for the HPLC run found in each fraction by the subunit labeling stoichiometry above.

(B) Identification of the Cysteines in the Auxiliary Disulfide. The thiol content of the E_{ox} form of each enzyme sample was determined as described in the preceding paper (Moore & Walsh, 1989), and only samples containing ca. 2 thiols/monomer were used for the following studies. Enzyme samples (20–40 nmol in 1.0 mL of 50 mM sodium phosphate, pH 7.5) were placed in double-side-armed cuvettes and were made anaerobic as described above, except that neither protocatechuate nor protocatechuate dioxygenase was added. After anaerobiosis had been achieved, 10 equiv of an anaerobic solution of [^{14}C]iodoacetamide (New England Nuclear, 2.41 mCi/mmol) was added to one side arm. Samples were titrated to the desired state of reduction with an anaerobic solution of NADPH from a repeating syringe. Titrations were followed spectrophotometrically, and iodoacetamide was tipped in after equilibrium had been attained (usually after ca. 20 min at 25 °C). After 1 h of incubation at 25 °C, cuvettese were opened to air, 50 μL of 50 mM cysteine was added, and samples were stored overnight at 4 °C. Enzyme samples that were not reduced with NADPH were treated similarly but were not made anaerobic. Proteins were precipitated by adding solid ammonium sulfate to 70% saturation followed by centrifuging at 13000 rpm for 20 min. Pellets were washed once with 80% ammonium sulfate and were then redissolved in 250 μL of 50 mM sodium phosphate, pH 7.5, plus 750 μL of 8 M urea. Remaining disulfides were reduced by incubating with 500 μM DTT at 37 °C for 1 h. The reduced thiols were exhaustively alkylated by adding cold iodoacetamide to a final concentration of 4 mM. After 1 h at 25 °C, 2-mercaptoethanol (10 μL) was added to stop the alkylation. Further treatment and analysis proceeded as described above.

Dithiol Reoxidation. DTT-treated $Ala_{10}Ala_{13}$ mutant enzyme (3–10 nmol) in 0.3 mL of 50 mM phosphate buffer, pH 7.3, and 0.3 mM EDTA, was denatured by adding 0.6 mL of 7.5 M guanidinium chloride in 65 mM Tris base containing 0.3 mM EDTA. Total thiols were assayed by reacting the enzyme at 4 °C with 125 nmol of 4,4′-dithiodipyridine (4-PDS) in 0.1 mL of 10% ethanolic 0.5 M phosphate buffer, pH 7.3/0.3 mM EDTA (total volume of 1 mL). The final pH was 7.0. The blank included 4-PDS. The 4-thiopyridone produced was calculated from the absorbance at 325 nm by using an extinction coefficient of 19800 M^{-1} cm^{-1} after subtracting the absorbance of the enzyme at that wavelength (Grassetti & Murray, 1967). The thiol titers measured with 4-PDS were the same as those measured with DTNB. A separate sample of 4.40 nmol of enzyme was reacted in a total volume of 1 mL of 50 mM phosphate buffer, pH 7.3/0.3 mM EDTA, at 4 °C with 4.38 nmol of 4-PDS. The 4-thiopyridone was measured at 325 nm with correction for oxidized enzyme. A separate sample of 14.7 nmol of enzyme was reacted anaerobically with 14.5 nmol of NADH and, after stabilization of the optical and fluorescence spectra, was further reacted with 30 nmol of 4-PDS. Disappearance of the charge-transfer band was observed at 530 nm and appearance of the 4-thiopyridone at 325 nm with correction for the oxidized enzyme absorbance. A stoichiometry of 2 mol of 4-thiopyridone produced to 1 mol of 4-PDS added was assumed to indicate the formation of 1 disulfide from 2 thiols as with DTNB (Flashner et al., 1972). This stoichiometry has been confirmed for 4-PDS in a model reaction with DTT.

Results

Electron Inventory: Evidence for a Third Redox-Active Group. We typically have found that initial additions of reductant in anaerobic titrations of mercuric reductase lead to spectral changes indicative of partial enzyme reduction followed by some reoxidation. Control titrations of free flavins suggested that this phenomenon was not due to oxygen contamination. Thus, we have reexamined the titrations of mercuric reductase with NADPH and dithionite in the presence of the oxygen scrubbing system, protocatechuate dioxygenase and protocatechuate, to avoid any uncertainty due to the presence of oxygen.

Titration of E_{ox} with NADPH. Representative spectra from the anaerobic titration of mercuric reductase with NADPH at 25 °C are shown in Figure 1. Each addition through the first equivalent initially gave spectral changes typical of formation of EH_2–$NADP^+$. This was followed by its decay to an E_{ox}–$NADP^+$ complex over a few minutes. Subsequent additions gave stable spectral changes. (See Scheme I for descriptions of these enzyme forms.) As shown in the titration curves (insets), equilibrium conversion of E_{ox} to the EH_2–$NADP^+$ complex (dot-dashed spectrum) required 2 equiv of NADPH. Further conversion to EH_2–NADPH required addition of excess NADPH. These results clearly show that another two-electron acceptor is reduced. Since NADPH must input electrons through the FAD, that acceptor must be reduced via the active site. Furthermore, the additional electron acceptor must be reduced in any stable EH_2 species.

Dithionite Titration of E_{ox}. To confirm the results of the NADPH titration and to preclude the possibility of yet another redox center of lower potential than that of enzyme-bound NADPH, we also repeated the dithionite titration of mercuric reductase. Figure 2 shows the results of such a titration in which both the UV–visible absorbance and fluorescence spectra were monitored. Again, the early additions gave spectral changes indicative of EH_2 formation followed by decay to an E_{ox} spectrum. Approximately 1.8 equiv of dithionite was required for maximal formation of EH_2, a stoichiometry essentially the same as seen with NADPH. [Similar results were previously reported by Fox and Walsh (1982) but were attributed to poor anaerobiosis.]

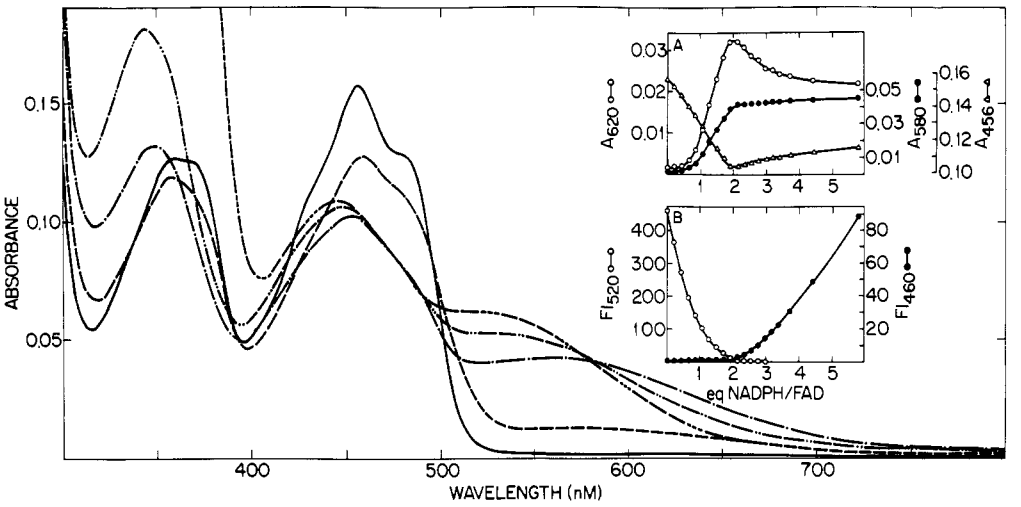


FIGURE 1: NADPH titration of KBr-dialyzed E_{ox}. Mercuric reductase (13.98 μM) was titrated with 0.90 mM NADPH at 25 °C. Spectra were recorded after each addition when no further absorbance changes occurred. Data are shown for (—) 0 equiv, (---) 1.07 equiv, (-·-) 2.13 equiv, (-··-) 2.98 equiv, and (-···-) 15.14 equiv of NADPH. The insets show (A) the absorbance at 620 (○), 580 (●), and 456 nm (△) and (B) the fluorescence emission of flavin at 520 nm, λ_{ex} = 456 nm (○), and of NADPH at 460 nm, λ_{ex} = 340 nm (●), as a function of equivalents of NADPH/FAD.

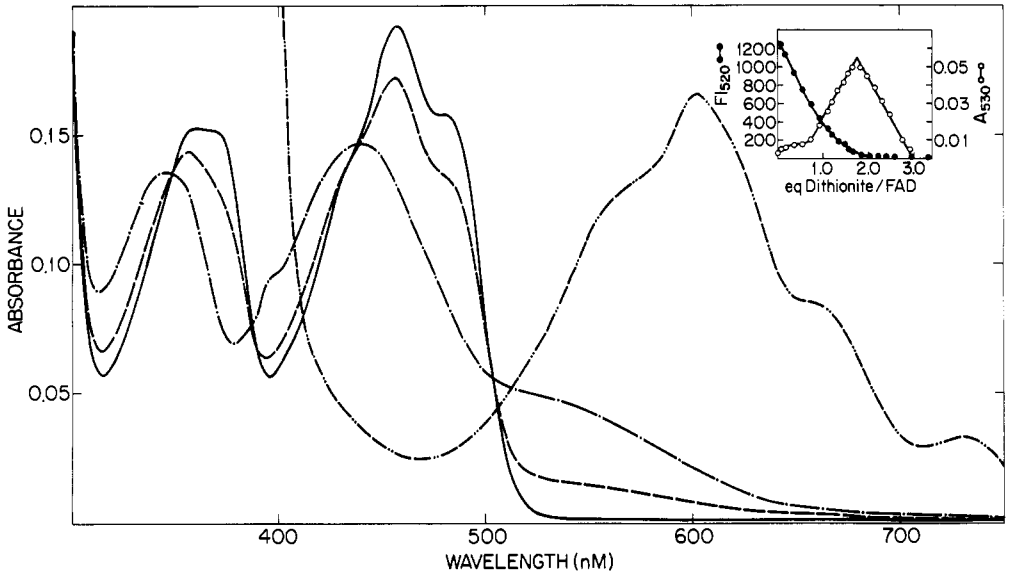


FIGURE 2: Dithionite titration of KBr-dialyzed E_{ox}. Mercuric reductase (17.1 μM) was titrated with 1.21 mM sodium dithionite at 4 °C in the presence of 100 μM methylviologen. Spectra were recorded after each addition when no further absorbance changes occurred at 456 nm. Data are shown for (—) 0 equiv, (---) 0.92 equiv, (-·-) 1.84 equiv, and (-··-) 3.31 equiv of dithionite. The inset shows the A_{530} (○) (corrected for absorbance of reduced methylviologen) and the flavin fluorescence at 520 nm, λ_{ex} = 456 nm (●), as a function of the equivalents of dithionite/FAD. Equilibrium absorbance due to methylviologen radical was seen only after addition of 1.65 equiv of dithionite.

The effect of reduction on the enzyme fluorescence was quite dramatic. Thus, while addition of 0.8 equiv gave at equilibrium no more than 16% of EH_2, on the basis of the absorption spectrum, the enzyme flavin fluorescence decreased to 44% of the starting value. This dramatic effect on a property of the FAD strongly suggests that the group reduced remains near the active site after reduction.

Identification and Properties of the Third Redox-Active Group. (A) Evidence That the Additional Redox-Active Group Is a Second Disulfide. With six other cysteine residues in the enzyme in addition to the active site disulfide, it seemed likely that the additional redox center might also be a disulfide. Thus, we examined the thiol titer of denatured enzyme before and after anaerobic reduction. As shown in Table I for wild-type enzyme, there are 4 more titratable thiols/monomer in EH_2–NADPH relative to E_{ox}, indicating that both the active site disulfide and an additional, auxiliary disulfide have been reduced.[2]

We next examined the possibility of obtaining enzyme oxidized in the active site, but containing a reduced auxiliary disulfide. As shown in Table I, DTT-treated E_{ox} (see Experimental Procedures), which is indistinguishable from fully oxidized E_{ox} by its absorption spectrum, contains 2 additional titratable thiols/monomer when denatured. Also, consistent with the results from the dithionite titration, this pretreated E_{ox} has only 42–45% of the fluorescence of the fully oxidized form of E_{ox}. The prereduced enzyme can also be generated by treatment with NAD(P)H and subsequent purification (data not shown).

[2] In a previous communication (Miller et al., 1986), we reported the presence of slowly titrating thiols in denatured mercuric reductase. We have since found that such behavior is an artifact of storing the enzyme at 0–4 °C for extended periods in the presence of mercaptoethanol. When the enzyme is prepared and stored as described under Experimental Procedures, all titratable thiols react rapidly with DTNB under denaturing conditions.

Table I: Thiol Content and Fluorescence Properties of Various Enzyme Forms

enzyme form[a]	thiols/ monomer	fluorescence[b] 2 °C	fluorescence[b] 25 °C
wild type			
E_{ox}	2.5	(6.3)	4.0
EH_2–NADPH	6.5[c]	0	0
DTT-treated E_{ox}	4.4	(2.6)	1.6
DTT-treated EH_2–NADPH	6.4[c]	0	0
$Ala_{10}Ala_{13}$			
E_{ox}	2.4	6.1	3.8
EH_2–NADPH	5.6	0	0
DTT-treated E_{ox}	3.6	4.2	2.4
DTT-treated EH_2–NADPH	5.7	0	0
$Ala_{558}Ala_{559}$			
E_{ox}	2.3	6.3	3.7
EH_2–NADPH	4.4	0	0
DTT-treated E_{ox}	3.5	5.8	3.5

[a] For each enzyme, native or mutant, the DTT-treated E_{ox} forms were derived directly from the E_{ox} forms by incubation with DTT followed by gel filtration through Sephadex G-25 to remove DTT just prior to the thiol and fluorescence measurements. [b] Fluorescence is reported relative to pure FAD at the same concentration. Values in parentheses were calculated for wild-type enzyme by using separately determined factors of 1.4 for the increase in enzyme fluorescence at 2 °C vs 25 °C and 0.88 for the decrease in FAD fluorescence at 2 °C vs 25 °C. [c] The total thiols/monomer reported here for the EH_2–NADPH complex is ca. 1.5 thiols lower than the expected value of 8. This is due to loss of some of the N-terminal cysteine residues by either proteolysis or overoxidation as discussed under Thiol Stability.

(B) Identification of the Thiols in the Auxiliary Disulfide. To determine which enzyme thiols are involved in the auxiliary disulfide, we have examined the thiol titer, NADPH consumption, and fluorescence properties of specific Cys to Ala mutants of mercuric reductase. For reference, there are 8 total cysteines in the enzyme: the active site pair ($Cys_{135}Cys_{140}$), the N-terminal pair ($Cys_{10}Cys_{13}$), the C-terminal pair ($Cys_{558}Cys_{559}$), Cys_{235}, and Cys_{403}. The thiol titer and fluorescence properties of untreated and DTT-treated E_{ox} and EH_2–NADPH forms of the $Ala_{10}Ala_{13}$ and $Ala_{558}Ala_{559}$ mutants are also summarized in Table I. The $Ala_{10}Ala_{13}$ mutant, which is essentially identical with wild-type enzyme in mercuric reductase activity, exhibited an increase of 3.2 thiols/monomer upon reduction from E_{ox} to EH_2–NADPH, with the total count of 5.6 only 0.4 less than the expected maximal value of 6. In this mutant, the extra disulfide is clearly present and can be reduced via the active site. As with wild-type enzyme, treatment with DTT gave an increase in the number of thiols/monomer in E_{ox}, which was accompanied by a decrease in the enzyme fluorescence to ca. 65% of the value for the untreated enzyme. By contrast, the $Ala_{558}Ala_{559}$ mutant, which has essentially zero mercuric reductase activity (Moore & Walsh, 1989) and consumes only 1 equiv of NADPH in forming a stable EH_2–$NADP^+$ species, showed only 2 thiols/monomer more in reduced than in oxidized enzyme. DTT treatment of this mutant resulted in an increase in the thiol titer for E_{ox} (presumably due to reduction of oxidized N-terminal thiols, see below), but in this case, there is only a marginal decrease in the enzyme fluorescence (final value ca. 93% of starting value). These results strongly suggest that Cys_{558} and/or Cys_{559} are part of the auxiliary reducible disulfide.

Thiol Stability. Since we are interested in comparing wild-type enzyme forms that contain different numbers of reduced thiols, it is important that we understand the stability of these thiols to air oxidation. Table II summarizes the stability of these thiols in E_{ox} and EH_2 forms of the wild-type enzyme as determined by thiol titrations of denatured enzyme.

Table II: Stability of Thiols in Wild-Type Enzyme upon Storage at 0–4 °C[a]

length of storage	enzyme form	incubation[b] time (min)	thiols/ monomer
freshly prepared	E_{ox}		3.8
	EH_2–NADPH	3–5	7.5
after 6–9 days	E_{ox}		2.6–2.9
	EH_2–NADPH	3–5	6.5
	EH_2 + NADH	45–60	7.4
after 3 weeks	E_{ox}		2.2
	EH_2 + NADH	45–60	5.6

[a] Stored in 50 mM potassium phosphate/0.3 mM EDTA, pH 7.3, in the absence of any thiol or pyridine nucleotide. [b] Incubation with NADPH or NADH was performed anaerobically at 25 °C for the indicated time prior to denaturation with guanidine hydrochloride.

As indicated, enzyme freshly treated with KBr usually contains between 3 and 4 thiols/monomer in E_{ox}; thus, at least 2 disulfides are present in this enzyme, one being the active site disulfide. As will be documented below, the $Cys_{558}Cys_{559}$ pair is quite susceptible to air oxidation; hence, it would appear that these residues are probably present as the other disulfide in freshly isolated, KBr-dialyzed enzyme. Note again in Table II that reduction of E_{ox} with NADPH gives an increase in the thiol titer of ca. 4 thiols/monomer in all cases and yields a final value close to the expected 8 thiols/monomer with freshly prepared enzyme. Upon storage at 0–4 °C, the thiol titer in E_{ox} decays to just over 2 thiols/monomer in ca. 2 weeks. Modeling of the mercuric reductase sequence on the structure of glutathione reductase (Thieme et al., 1981) predicts Cys_{235} and Cys_{403} to be spatially separated and buried in the folded protein; hence, they are likely to be the 2 thiols resistant to oxidation. The slow disappearance of thiols then (Table II, rows 1, 3, and 6) is presumably due to oxidation of the N-terminal cysteine pair or loss due to proteolysis of the first 85 amino acids, which occurs spontaneously upon storage at 4 °C (Fox & Walsh, 1983). Reduction of a third disulfide (presumably at $Cys_{10}Cys_{13}$) occurs both by extended incubation with pyridine nucleotide (see Table II) and by treatment of E_{ox} with DTT (see Table I, $Ala_{558}Ala_{559}$ DTT-treated E_{ox}). However, neither treatment can reduce any group that has been oxidized beyond the sulfenic acid oxidation state. Thus, after longer storage at 0–4 °C, extended incubation with pyridine nucleotide shows only the increase of 4 thiols/monomer in EH_2 relative to E_{ox} for the reduction of the active site and auxiliary disulfides (Table II), and DTT treatment shows an increase of only 2 thiols/monomer in treated E_{ox} relative to untreated E_{ox} due to the reduction of the auxiliary disulfide (Table I, wild type). Note that DTT does reduce the active site disulfide, as recognized by the typical EH_2 spectrum, but that the disulfide is reformed by air oxidation via the flavin during the gel filtration to remove DTT.

Figure 3 shows the time course of the spontaneous oxidation of wild-type mercuric reductase which had been treated with DTT and the correlation of the flavin fluorescence of the enzyme with the oxidation state of the enzyme thiols. Freshly prepared enzyme (see Figure 3 legend) contained 6.5 thiol residues per protein-bound FAD and a fluorescence at 2 °C 2.7-fold more intense than that of free FAD. On storage in ice under aerobic conditions, the thiol titer decayed as shown, to reach a fairly stable value over 1 week of 3.2–3.3 thiols/monomer. During the same time, the fluorescence intensity increased to 5.75 times that of FAD. The absorption spectrum remained unchanged. The fluorescence excitation and emission spectra changed only in intensity. The fluorescence excitation spectrum was essentially identical with that of the absorption spectrum.

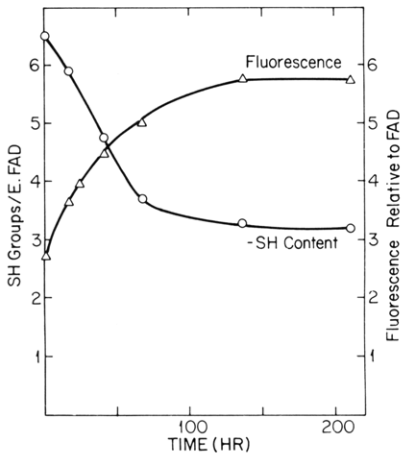


FIGURE 3: Effect of thiol oxidation on the fluorescence of mercuric reductase. The apoprotein of mercuric reductase was obtained by dialysis at 4 °C vs 2 M KBr, 0.1 M sodium acetate, and 0.3 mM EDTA, pH 4.5, to remove the FAD, followed by dialysis vs 50 mM potassium phosphate buffer, pH 7.0, containing 0.3 mM EDTA, to remove KBr. The apoprotein was then incubated for 30 min with 6 mM DTT at 0 °C, mixed with 10^{-4} M FAD, and incubated for a further 15 min at 0 °C and then for 10 min at 25 °C. It was then separated from excess FAD and from DTT by passage through G-25 equilibrated with 50 mM potassium phosphate, pH 7, containing 0.3 mM EDTA. The reconstituted enzyme had a spectral ratio, A_{273}/A_{457} of 6.57, marginally higher than that of 6.3 reported for the enzyme as isolated (Fox & Walsh, 1982). The reconstituted enzyme was stored on ice, and at intervals, its fluorescence was recoreded and a 0.1-mL sample assayed for thiol content by addition to 0.9 mL of 6 M guanidine hydrochloride in 50 mM phosphate, pH 7.5, 25 °C, followed within 1 min by 10 μL of 10^{-2} M DTNB. The A_{412} was read vs a blank without enzyme treated in the same manner. The absorbance increase in the enzyme sample was in all cases complete within 90 s of adding the DTNB.

[^{14}C]Iodoacetamide Labeling. While the above results clearly implicate Cys_{558} and/or Cys_{559} in the auxiliary disulfide, they do not allow the exact determination of which thiols are involved. The auxiliary disulfide could be formed either between Cys_{558} and Cys_{559} or between one of these and either Cys_{235} or Cys_{403}. Two points of evidence strongly favor the first possibility. Modeling of the mercuric reductase sequence to the structure of human glutathione reductase (as described above) predicts that both Cys_{235} and Cys_{403} would be located far away from the active site and likely to be inaccessible for disulfide formation with other enzyme thiols. Second, previous [^{14}C]iodoacetamide labeling studies (Fox & Walsh, 1983) showed that while no label was incorporated into wild-type E_{ox}, enzyme reduced with dithionite to the EH_2 level reacted with 1 equiv of iodoacetamide. Of this 1 equiv, 80% was found in the active site peptide and 20% in the C-terminal peptide. In order to obtain more evidence as to which residues are involved in the auxiliary disulfide, we undertook a more extensive [^{14}C]iodoacetamide labeling study using various oxidized and reduced forms of wild-type and mutant mercuric reductases.

Figure 4 shows the results of labeling mercuric reductase with [^{14}C]iodoacetamide under denaturing conditions. The five cysteine–containing tryptic fragments of wild-type mercuric reductase resolved by HPLC could be identified by comparing the wild-type elution pattern with those obtained for the mutant enzymes. Peak 1 corresponds to the C-terminal peptide ($Cys_{558}Cys_{559}$) as evidenced by its absence from the $Ala_{558}Ala_{559}$ digest. Peak 3 contains the active site pair ($Cys_{135}Cys_{140}$), since that peak is not present in the $Ala_{135}Ala_{140}$ digest. Peak 2 can be assigned to the peptide containing the N-terminal cysteine pair ($Cys_{10}Cys_{13}$) because of its progressive disappearance as one goes from 50% to 90% to 100% "clipped"

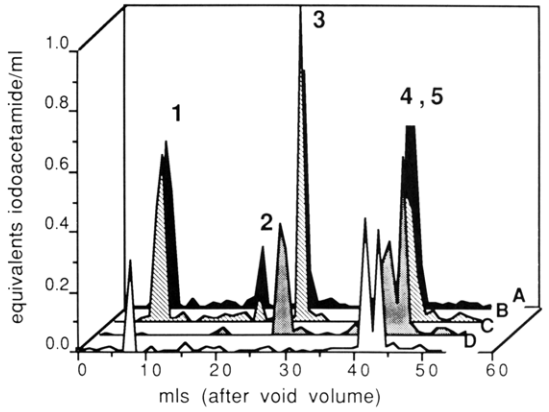


FIGURE 4: Identification of[^{14}C]iodoacetamide-labeled peptides from exhaustively reduced wild-type and mutant mercuric reductases. Protein samples were exhaustively reduced under denaturing conditions and reacted with [^{14}C]iodoacetamide as described under Methods. After tryptic digestion, peptides (>10 000 cpm) were separated by HPLC and analyzed for radioactivity as described under Methods. Samples are as follows: (A) 50% "clipped" wild type, (B) 90% clipped wild type, (C) 100% clipped $Ala_{558}Ala_{559}$, and (D) 100% clipped $Ala_{135}Ala_{140}$.

enzymes. [Fox and Walsh (1983) showed that partial proteolysis of wild-type mercuric reductase in vitro results in a clipped form of the enzyme which is catalytically active but lacks the first 85 N-terminal residues.] Lastly, as peaks 4 and 5 are present in all four digests, they must necessarily correspond to the peptides containing the unpaired cysteines (Cys_{235}, Cys_{403}).

We next examined the labeling stoichiometry for each peptide upon reaction of the native forms of oxidized and NADPH-reduced wild-type, $Ala_{135}Ala_{140}$, and $Ala_{558}Ala_{559}$ enzymes with [^{14}C]iodoacetamide. The $Ala_{558}Ala_{559}$ mutant was chosen because we knew from the thiol data above and from the NADPH and dithionite titration data with this mutant (Moore & Walsh, 1989) that the auxiliary disulfide has been disrupted in this protein by removal of one or both of the residues involved. Conversely, the $Ala_{135}Ala_{140}$ mutant, which lacks the active site disulfide, was chosen in order to examine the need for this moiety in the reduction of the auxiliary disulfide. The enzymes were first assayed for thiol content to ensure that each sample contained only 2 thiols/monomer (the residual thiols as described above), i.e., that the oxidized forms of wild-type enzyme and the $Ala_{135}Ala_{140}$ mutant did contain an intact auxiliary disulfide. E_{ox} samples were reacted aerobically with [^{14}C]iodoacetamide. Reduced samples were produced by anaerobic addition of NADPH, rather than dithionite as in Fox and Walsh (1983). The results of tryptic hydrolysis and specific activity determination are summarized in Table III.

Comparison of the radioactivity incorporated into the cysteine-containing peptides from wild-type E_{ox} (auxiliary disulfide), E_{act}–$NADP^+$, and EH_2–$NADP^+$ (auxiliary dithiol) indicates that all four groups of peptides become more reactive toward iodoacetamide upon reduction of enzyme with NADPH. However, we have already shown above that Cys_{10} and Cys_{13} do not participate in the auxiliary disulfide. The 0.2-equiv increase in labeling at this site in the EH_2–$NADP^+$ complex correlates well with the excess 0.1 equiv of NADPH present in the reaction mixture and is probably due to dithiol/disulfide interchange with the redox-active and/or auxiliary dithiols during the prolonged iodoacetamide incubation period. This is consistent with the data presented in Table II, where after 1–2 weeks of storage, incubation of E_{ox} with NAD(P)H for short periods of time gives reduction of

Table III: [^{14}C]Iodoacetamide Labeling Stoichiometry of Cysteine-Containing Peptides from Wild-Type and Mutant Mercuric Reductases

enzyme	oxidation state	equiv of NADPH	labels/monomer			
			$Cys_{10}Cys_{13}$	$Cys_{135}Cys_{140}$	$Cys_{558}Cys_{559}$	Cys_{235}, Cys_{403}[a]
wild type	E_{ox}	0.0	0.08	0.04	0.02	0.01
	E_{act}–$NADP^+$	1.1	0.05	0.25	0.16	0.06
	EH_2–$NADP^+$	2.1	0.28	0.50	0.50	0.08
$Ala_{558}Ala_{559}$	E_{ox}	0.0	0.00[b]	0.01		<0.01
	EH_2–$NADP^+$	1.1	0.00[b]	1.06		0.07
$Ala_{135}Ala_{140}$	E_{ox}	0.0	0.06		0.01	<0.01
	E_{ox} + NADPH	1.4	0.04		0.01	<0.01

[a] While the three peptides containing cysteine pairs are well separated by HPLC (Figure 4), which facilitates the determination of their individual labeling stoichiometries, the peptides containing Cys_{235} and Cys_{403} are not. Thus, their stoichiometries are given as a combined labeling score. [b] These enzyme samples were completely "clipped", accounting for the lack of N-terminal labeling.

only the active site and auxiliary disulfides, but extended incubation shows reduction of yet another disulfide. Similarly, the 0.07-equiv increase in labeling of the unpaired cysteines, Cys_{235} and Cys_{403}, could be indicative of involvement by one of them in the auxiliary disulfide. However, the $Ala_{558}Ala_{559}$ enzyme, which lacks the auxiliary disulfide, shows the same intensification of unpaired cysteine labeling upon reduction from E_{ox} to EH_2–$NADP^+$. Thus, this increased reactivity is likely to be due to a conformational change, making one or both thiols more solvent accessible. Given that the greatest increase in labeling occurs in the redox-active and C-terminal peptides, we feel that these data strongly support the hypothesis that the auxiliary disulfide is formed between Cys_{558} and Cys_{559}.

There are several other points brought out by the iodoacetamide labeling data. The active site disulfide is required for auxiliary disulfide reduction by NADPH, as can be seen from data for the $Ala_{135}Ala_{140}$ mutant. This is consistent with the spectral observations that addition of 1 equiv of NADPH to wild-type E_{ox} initially reduces the active site disulfide, followed by a slower reoxidation of this dithiol and reduction of the auxiliary disulfide. Thus, the auxiliary disulfide reduction must occur by a dithiol/disulfide interchange mechanism, rather than by direct reduction from the flavin, requiring that Cys_{558} and Cys_{559} be in close proximity to Cys_{135} and Cys_{140}.

The juxtapositioning of the two cysteine pairs is further illustrated by the fact that although there are 4 thiols per active site in the wild-type EH_2–$NADP^+$ complex, only 1 equiv of iodoacetamide is taken up per active site, split equally between the redox-active and C-terminal cysteines. Such stoichiometry cannot be attributed to limiting iodoacetamide, since 10 equiv were used in labeling, but more likely results from steric constraints within the active site. Presumably, the 4 thiols are close enough that reaction of one of them with iodoacetamide inhibits further reaction by another with a second iodoacetamide molecule. Consistent with this, the EH_2–$NADP^+$ complex of the $Ala_{558}Ala_{559}$ mutant also reacts with only 1 equiv of iodoacetamide per active site, with all radioactive label associated with the active site disulfide.

Oxidation of the Auxiliary Dithiol Pair with Stoichiometric 4,4′-Dithiodipyridine. As a test of the ease with which the $Cys_{558}Cys_{559}$ thiol pair is oxidized to a disulfide, we have examined the reaction of the $Ala_{10}Ala_{13}$ mutant enzyme with a stoichiometric quantity of the specific thiol reagent 4,4′-dithiodipyridine (4-PDS). (The $Ala_{10}Ala_{13}$ mutant was chosen for this study to avoid any ambiguities that might arise in the presence of the N-terminal thiols.) If two thiols are properly juxtaposed for disulfide formation, they will react with 1 mol of DTNB or 4-PDS to yield 2 mol of 2-nitro-5-thiobenzoate or 4-thiopyridone, respectively (Flashner et al., 1972). When the DTT-treated E_{ox} form of $Ala_{10}Ala_{13}$ is combined with stoichiometric 4-PDS, 1.6 equiv of 4-thiopyridone per FAD was released, indicating formation of 0.8 disulfide/monomer. When another sample of this DTT-treated enzyme was reduced anaerobically to EH_2 with 0.98 equiv of NADH, 3.94 equiv of 4-thiopyridone was produced upon addition of 2 equiv of 4-PDS. This indicated formation of 1.97 disulfides/monomer. Thus, both the active center dithiol pair ($Cys_{135}Cys_{140}$) produced by NADH reduction and the auxiliary dithiol pair can be reoxidized by reaction with 4-PDS. Reoxidation of the active center dithiol by 4-PDS is an order of magnitude faster than the same reaction involving the auxiliary dithiol. The rate of oxidation of DTT by 4-PDS is approximately the same as the rate for the auxiliary dithiol (data not shown).

Effect of Redox State of the Auxiliary Dithiol/Disulfide on Catalytic Activity. Having established the presence of the auxiliary disulfide and its reducibility via the active site, the question remains as to its role in catalysis: Are both the auxiliary disulfide and dithiol forms of the enzyme catalytically relevant or just one of them? To answer this, we have examined both the pre-steady-state reduction of the enzyme with NADPH and the steady-state initial velocity behavior of enzyme with a defined auxiliary disulfide/dithiol redox state.

(A) Pre-Steady-State Reduction of Mercuric Reductase with NADPH. From previous aerobic rapid reaction studies at 5 °C, Sahlman et al. (1984) reported that formation of EH_2–$NADP^+$ requires only 1 equiv of NADPH/FAD and formation of EH_2–NADPH is complete with only 2 equiv of NADPH/FAD. They further reported a slow decay of the final EH_2–NADPH complex under aerobic conditions which was attributed primarily to reaction with oxygen (Sahlman & Lindskog, 1983). We have reexamined the reduction kinetics anaerobically to determine, first, whether we see uptake of a second equivalent of NADPH by the auxiliary disulfide form of the enzyme but not by the dithiol form and, if so, whether the rate of reduction is consistent with catalysis. Figure 5 shows kinetic traces at 525 nm for reaction of 2 equiv of NADPH with either untreated E_{ox} (auxiliary disulfide) or DTT-treated E_{ox} (auxiliary dithiol) at both 4 and 25 °C. At 4 °C, formation of EH_2–NADPH is complete in <1 s, but with untreated enzyme, this complex slowly decays to an EH_2–$NADP^+$ complex (k = 0.003 s^{-1}). At 25 °C, this reaction becomes easily observable and is complete in ca. 4 min (biphasic trace with rate constants of 0.135 s^{-1} and 0.02 s^{-1}). DTT-treated enzyme shows only formation of the EH_2–NADPH complex with no significant decay over the same time periods at either 4 or 25 °C. These results indicate that the slow consumption of a second equivalent of NADPH by the auxiliary disulfide form of the enzyme is indeed due to reduction of the disulfide. However, the rate of this reduction is far too slow to be catalytically relevant. [Turnover is reported to be 13 s^{-1} at 37 °C (Fox & Walsh, 1982) and 9–13

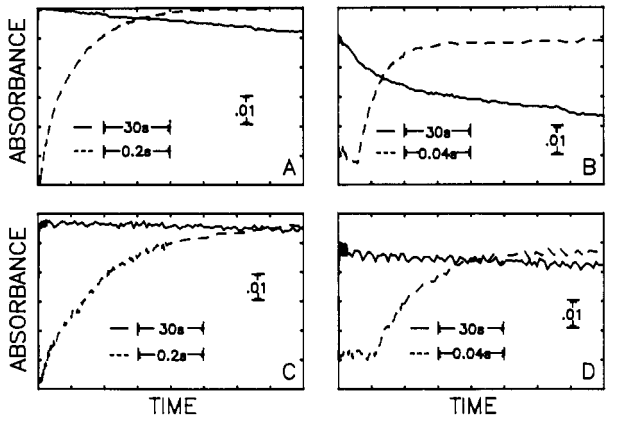


FIGURE 5: Reaction of NADPH with untreated and DTT-treated E_{ox}. In each case mercuric reductase was mixed in the stopped-flow spectrophotometer with 2 equiv of NADPH and the absorbance at 525 nm observed. Kinetics are shown for untreated enzyme (9 μM final concentration) at (A) 4 °C, 0.8 s (---) and 120 s (—), and (B) 25 °C, 0.16 s (---) and 120 s (—), and for DTT-treated enzyme (8.4 μM final concentration) at (C) 4 °C, 0.8 s (---) and 120 s (—), and (D) 25 °C, 0.16 s (---) and 120 s (—).

s^{-1} at 25 °C (Sandstrom & Lindskog, 1987).] Thus, cycling of the auxiliary disulfide between reduced and oxidized states cannot occur during catalysis.

In addition to the absence of the slow decay phase, the DTT-treated enzyme exhibits different rates of reduction by NADPH than does the untreated enzyme. At 4 °C the untreated enzyme shows rates of 45.9 s^{-1} and 8.6 s^{-1} for formation of EH_2–$NADP^+$ and EH_2–NADPH, respectively, similar to the values reported by Sahlman et al. (1984). However, DTT-treated enzyme shows rates of 117 ± 13 s^{-1} and 5.5 ± 0.3 s^{-1} for the same phases. At 25 °C formation of the EH_2–NADPH complex from EH_2–$NADP^+$ occurs at a rate of 82.4 s^{-1} with untreated enzyme and at 38.1 ± 1.3 s^{-1} with DTT-treated enzyme (formation of EH_2–$NADP^+$ is too fast to measure at this temperature). Two points should be made: (1) the pretreated enzyme is more readily reduced by NADPH, suggesting that the prereduced enzyme may be in a more active form (see below); (2) $NADP^+$ dissociation, which is proposed to limit the formation of EH_2–NADPH (Sahlman et al., 1984) during turnover, is slower from four-electron-reduced EH_2 (generated from DTT-treated E_{ox}) than it is from two-electron reduced EH_2 (generated from untreated E_{ox}).

(B) Steady-State Initial Velocity Studies. During the course of our studies, Sandstrom and Lindskog (1987) reported that isolated, KBr-dialyzed mercuric reductase shows a steady-state initial velocity near zero and undergoes an activation during the course of turnover. Preincubation of the enzyme with NADPH or 30 mM cysteine eliminates the lag, generating a more active form of the enzyme. Hence, they have designated the two forms nonactivated and activated enzyme, respectively. While they were unable to detect a difference in the thiol titer of the enzyme upon activation,[3] their data suggested to us that the auxiliary disulfide form of the enzyme may correspond to nonactivated enzyme with the auxiliary dithiol form then corresponding to the activated enzyme. Thus, we have briefly examined the steady-state initial velocity behavior of the enzyme with a defined auxiliary disulfide/dithiol redox state using the stopped-flow spectrophotometer to monitor the absorbance at 340 nm. With dilute enzyme in one syringe and NADPH, $HgCl_2$ and thiol (2-mercaptoethanol or cysteine) in the other syringe, the auxiliary disulfide form of the enzyme does exhibit very short lags during steady-state assays as previously reported (Miller et al., 1986; Sandstrom & Lindskog, 1987), whereas the auxiliary dithiol form of the enzyme shows no lag under identical assay conditions. Additionally, the dithiol form of the enzyme exhibits a higher initial velocity than the fastest steady-state rate attained by the disulfide form of the enzyme. Premixing of the dithiol form of the enzyme with NADPH had no effect on its initial velocity behavior, whereas premixing the disulfide form of the enzyme with NADPH eliminated the lag as was reported by Sandstrom and Lindskog (1987). Hence, we conclude that activation of the enzyme does indeed involve reduction of the auxiliary disulfide.

[3] Sandstrom and Lindskog have recently confirmed our finding of the appearance of 2 additional thiols/monomer upon anaerobic activation with NADPH (personal communication).

Discussion

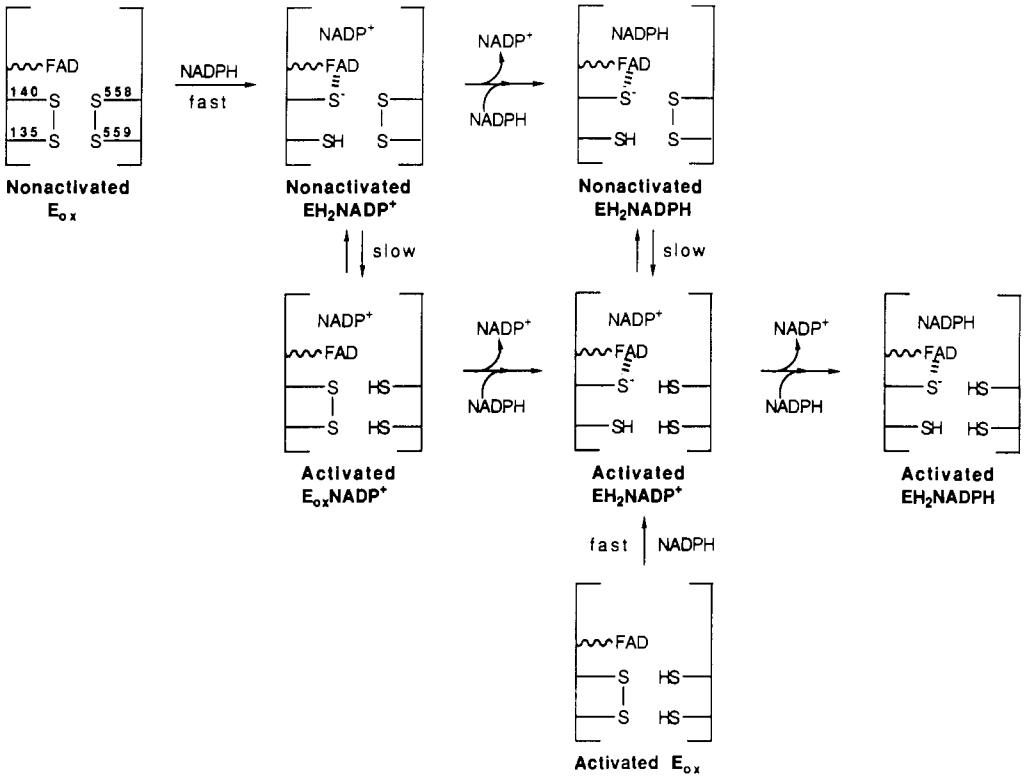
Mercuric reductase is highly homologous with lipoamide dehydrogenase and glutathione reductase in all domains, showing identity at 10–12 of 14 positions in the peptide centered at the active site disulfide. The spectral properties of the EH_2–NADPH and EH_2–$NADP^+$ complexes of mercuric reductase are virtually identical with those of the analogous complexes of the pyridine nucleotide disulfide oxidoreductases. All of these enzymes bind Hg(II) at the active site dithiol; however, only mercuric reductase has the ability to reduce Hg(II) at a useful rate [see Moore and Walsh (1989)]. What feature of this enzyme allows it to reduce Hg(II)?

Brown et al. (1983) suggested that the C-terminal cysteine pair (558, 559) may be involved in binding of Hg(II) at the active site. This proposal derives from the assumption that with the extensive sequence homology between glutathione reductase and mercuric reductase the proteins are likely to fold similarly. Since glutathione reductase has the C-terminus of one monomer of the active dimeric protein folded into the active site of the other monomer to provide a histidine residue essential for catalysis, it seems reasonable to propose that the C-terminal cysteines of one subunit of mercuric reductase may be similarly folded into the active site of the other subunit. A second consideration is their complete conservation in all mercuric reductases sequenced to date (Brown et al., 1983; Misra et al., 1985; Laddaga et al., 1987; Griffin et al., 1987), coupled with the fact that the other members of this enzyme family which cannot reduce Hg(II) lack analogous cysteine residues. Finally, the most compelling piece of evidence for the importance of Cys_{558} and/or Cys_{559} in catalysis is that the $Ala_{558}Ala_{559}$ mutant has 1000-fold lower Hg(II) reductase activity than wild-type enzyme (Moore & Walsh, 1989).

In the current work we have demonstrated that Cys_{558} and Cys_{559} are in close communication with the active site. First, we have shown the existence of an additional, previously unreported disulfide, which is reducible with NADPH via the flavin and redox-active disulfide or directly by treatment of the enzyme with DTT. Second, by thiol titrations of the $Ala_{10}Ala_{13}$ and $Ala_{558}Ala_{559}$ mutant enzymes, we have shown that this disulfide is not composed of either Cys_{10} or Cys_{13} but must involve at least one of the C-terminal cysteines. Last, by examining the pattern of [^{14}C]iodoacetamide incorporation into oxidized and NADPH-reduced forms of the wild-type, $Ala_{558}Ala_{559}$, and $Ala_{135}Ala_{140}$ mutant enzymes, we have been able to show that the second disulfide is indeed formed between Cys_{558} and Cys_{559}.

While disulfides between adjacent cysteines are unusual in proteins, they are by no means unknown. Their rarity probably stems from the fact that such disulfides form eight-membered

Scheme I: Reactions of NADPH with the Activated and Nonactivated Forms of Mercuric Reductase



rings, constraining the intracysteinyl peptide bond to be in the cis rather than its preferred trans orientation (Capasso et al., 1977). These conformational constraints make adjacent half-cystinyl disulfides perfect candidates for molecular switching devices; such a role seems to be played by the disulfide between Cys_{192} and Cys_{193} in the nicotinic acetylcholine receptor from electric eel (Kao & Karlin, 1986). Other adjacent disulfides have been found in the γ-subunit of bovine transducin (Ovichinnikov et al., 1985) and the cyclic fungal pentapeptide malformin A (Bodanszky & Stahl, 1974).

The close association of the second disulfide with the active site is indicated by several lines of evidence. First, while its redox state has no detectable effect on the UV–visible absorbance spectrum, conversion of the second disulfide to the dithiol dramatically diminishes the fluorescence intensity of the enzyme flavin. By contrast, reduction of a disulfide in the N-terminal domain (see Table I, $Ala_{558}Ala_{559}$, DTT-treated E_{ox}), which is not part of the active site (Fox & Walsh, 1983), has little effect on enzyme fluorescence. Thus, we have designated the $Cys_{558}Cys_{559}$ disulfide as the "auxiliary" disulfide. Second, reduction of the auxiliary disulfide by NADPH must occur via dithiol/disulfide interchange with the active site redox-active cysteine pair, $Cys_{135}Cys_{140}$, as evidenced by the lack of iodoacetamide labeling of the C-terminal cysteines upon reduction of the $Ala_{135}Ala_{140}$ mutant with NADPH. Such evidence of communication strongly supports the hypothesis that either one or both of the cysteines in the auxiliary dithiol are close enough to the active site to participate in Hg(II) binding as proposed by Brown et al. (1983). Further support comes from the iodoacetamide labeling data showing that fully reduced wild-type enzyme can only react with one iodoacetamide per active site (see Table III). Thus, the four thiols must be close enough such that carboxymethylation of one introduces steric constraints on reaction of the others with iodoacetamide. Combining all of the above data, we propose the pathways shown in Scheme I for reaction of NADPH with the nonactivated and activated forms of the enzyme.

We have also examined the kinetics of enzyme reduction by NADPH and have shown that reduction of the auxiliary disulfide is too slow to occur actively during catalysis. However, initial velocity measurements performed with enzyme of defined disulfide/dithiol redox state indicate that reduction of the auxiliary disulfide is a necessary priming process for catalysis. This suggests that, under the normal reducing conditions of the bacterial cell, the auxiliary dithiol pair is always in its fully activated form for binding and reduction of Hg(II) (see below).

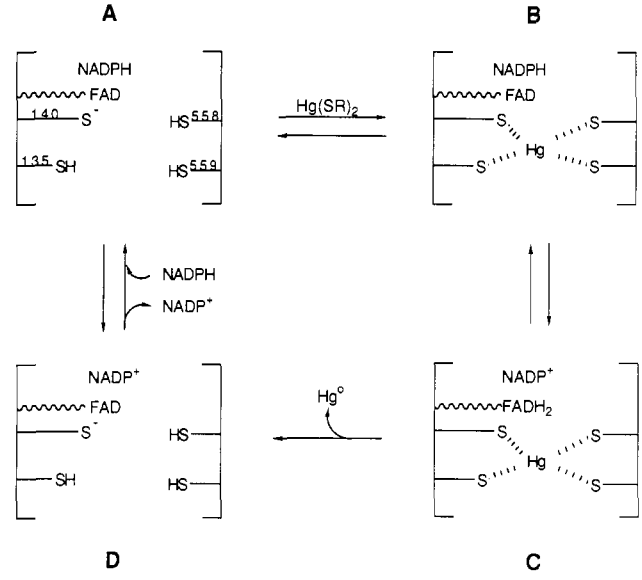
The above conclusions are consistent with the finding of Sandstrom and Lindskog (1987) that purified mercuric reductase can be activated for Hg(II) reduction by prior incubation of the enzyme with NADPH and/or cysteine. While they were unable to demonstrate that activation involved the reduction of a disulfide,[3] we feel confident that enzyme with the C-terminal disulfide in the reduced state is the "activated" enzyme reported by Sandstrom and Lindskog (1987). Given the precedence in nomenclature, we shall therefore refer to oxidized enzyme containing an auxiliary dithiol as "activated E_{ox}" or "E_{act}" and that containing the auxiliary disulfide as "nonactivated E_{ox}". Such nomenclature has the advantage of preserving the E_{ox}, EH_2, and EH_4 notation for the oxidation state of the flavin and redox-active disulfide, while including the oxidation state of the newly characterized auxiliary disulfide.

Given that the auxiliary dithiol is required for normal catalytic reduction of Hg(II) by mercuric reductase, the question arises as to the exact role of this dithiol in catalysis. A consideration of the thermodynamics of Hg(II) reduction in the presence of thiols suggests one plausible role of the auxiliary dithiol. The reduction potential for free Hg(II) is ca. +850 mV. However, thiols typically form 2-coordinate complexes with Hg(II) having association constants (K_a) in the range of 10^{38}–10^{45}. At the low end of this range are complexes with two separate ligands such as $Hg(Cys)_2$ (Stankovich & Bard, 1977), while at the high end are di-

thiol-chelated complexes such as Hg(2,3-dimercaptopropanesulfonate) (K_a = $10^{42.2}$) and Hg(2,3-dimercaptopropan-1-ol) (K_a = $10^{44.8}$) (Casas & Jones, 1980). The effect of the high K_a values for these complexes is to lower the Hg(II) reduction potential to values in the range of −270 mV for $Hg(Cys)_2$ to between −390 and −475 mV for the chelated complexes. Comparison of the value for $Hg(SR)_2$ to the reduction potential for NADPH (−320 mV) indicates that the overall reaction catalyzed by mercuric reductase, eq 1, is thermodynamically favorable. However, if a bis-chelated Hg(II) complex is formed with just the active site dithiol, reduction of the enzyme-bound Hg(II) could become thermodynamically unfavorable due to the greater stability of the chelated complex. As will be elaborated below, we believe that this potential thermodynamic stabilization via bis chelation of Hg(II), and its anticipated attenuation of enzymatic rate, is avoided by mercuric reductase through the participation of Cys_{558} and/or Cys_{559}. As demonstrated by stopped-flow spectrophotometric experiments, formation of bis-chelated Hg(II) bound to the $Cys_{135}Cys_{140}$ dithiol results in no observable reduction of Hg(II). Thus, when mixed with only 1 equiv of $Hg(CN)_2$ and excess NADPH, both the $Ala_{558}Ala_{559}$ mutant E_{ox} and the wild-type nonactivated E_{ox} undergo rapid reduction of the active site disulfide and binding of Hg(II) to form identical EH_2–NADPH–Hg(II) complexes. These complexes are resistant to Hg(II) reduction even though the FAD appears to be partially reduced (Miller et al., 1987; Moore et al., 1987). These enzymes, which lack the auxiliary dithiol (just like glutathione reductase and lipoamide dehydrogenase), have no problem binding Hg(II) at the active site dithiol, but they cannot reduce it in the dithiol chelated complex at a catalytically relevant rate. Thus, we suggest that mercuric reductase utilizes the auxiliary dithiol to prevent formation of this highly stabilized (i.e., low-potential) dithiol chelated Hg(II). As our working hypothesis, we propose that the auxiliary dithiol participates with the active site thiol/thiolate pair to provide a tri- or tetracoordinate Hg(II) complex at the active site.

What advantage should a polycoordinate thiol–Hg(II) complex have over a 2-coordinate complex? In general, linear 2-coordinate complexation predominates in the chemistry of Hg(II). Basically, this is due to hybridization of the $5d_{z^2}$ and the 6s orbitals (allowed by their small energy difference) followed by further hybridization of the empty hybrid with a p orbital to yield two bonding orbitals in a linear configuration (Dunitz & Orgel, 1960). Thiols, in particular, provide excellent orbital overlap in such 2-coordinate complexes, manifested by the very high association constants and low reduction potentials for these species. When additional ligands are placed around Hg(II), the resulting complex is energetically destabilized relative to the 2-coordinate complex, primarily due to unfavorable interactions between ligand electrons and those on Hg(II). With the greater electronic repulsion, each of the Hg–S bonds in the polycoordinate complex should be more ionic in character, resulting in more positive charge being localized on the Hg(II) than in the 2-coordinate complex and, hence, a higher reduction potential. Such polycoordnate Hg(II) complexes have been shown to occur, at least transiently, during thiol ligand exchange reactions on 2-coordinate Hg(II) complexes (Rabenstein & Fairhurst, 1975), which clearly indicates their destabilization relative to the 2-coordinate species. From a kinetic standpoint, it is uncertain how polycoordination vs. 2-coordination would affect the energy of the transition state for reduction. However, if the transition-state energies are similar for reduction of the two complexes, raising the ground-state energy for bound Hg(II) by making it polycoordinate would lower the energy of activation for reaching the transition state and thereby increase the rate of reduction. Such ground-state destabilization is a common theme in enzyme catalysis (Fersht, 1977).

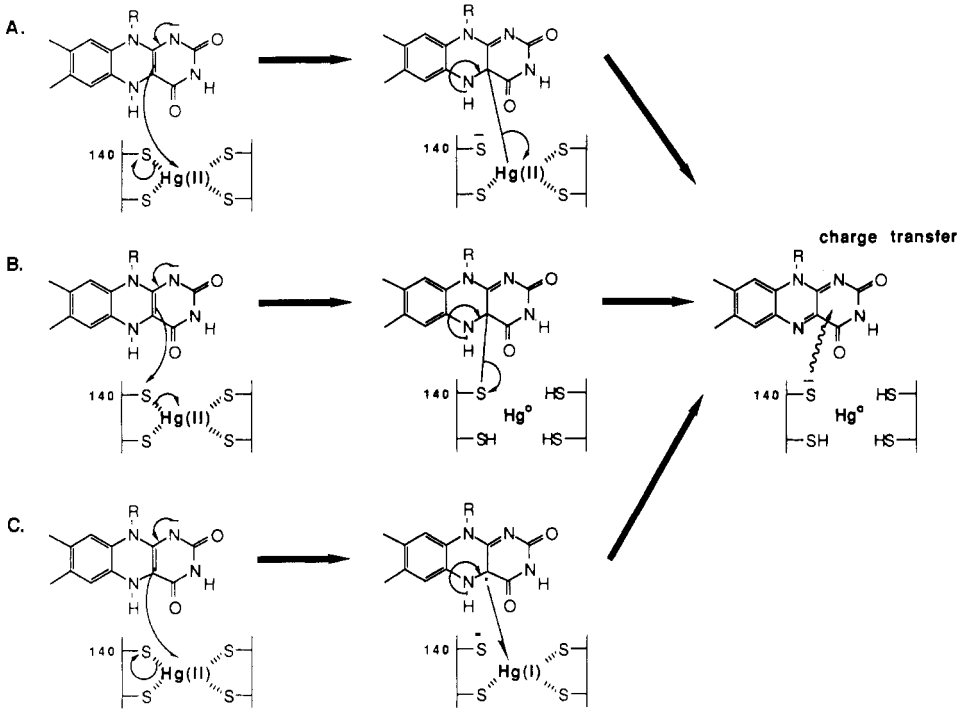
Scheme II: Modified Minimal Catalytic Mechanism for Wild-Type Mercuric Reductase[a]



[a] Although indicated as tetracoordinate, the Hg(II) complex in species B and C may be tricoordinate. Additionally, the protonation state of the several thiol ligands is uncertain although they are depicted as thiolates.

A modified version of our minimal catalytic mechanism (Miller et al., 1986), incorporating the ideas presented here and those developed in the preceding papers (Distefano et al., 1989; Moore & Walsh, 1989), is presented in Scheme II. In our earlier work, where we demonstrated that uncomplexed EH_2 could bind, but not reduce, Hg(II), we concluded that rather than cycling between E_{ox} and EH_2, where the active site dithiol undergoes a reversible oxidation/reduction during catalysis (as in glutathione reductase and lipoamide dehydrogenase), mercuric reductase cycles between EH_2–$NADP^+$ and EH_2–NADPH (i.e., an enzyme form containing 4 electron equiv). In this case, the active site dithiol need not provide the reducing power for the Hg(II), but just a binding site (Miller et al., 1986). The work of Schultz et al. (1985) and of Distefano et al. (1989) strengthens the hypothesis that the active site dithiol in mercuric reductase serves to bind rather than to reduce Hg(II). This conclusion is also consistent with the observation by Stankovich and Bard (1977) that $Hg(Cys)_2$ undergoes a reversible oxidation/reduction at the mercury electrode in which the Hg(II) rather than the thiols undergoes oxidation/reduction. In the current proposal, the above ideas are the same but the viable enzyme–Hg(II) complex contains Hg(II) liganded to 3–4 enzyme thiols. In addition to the inclusion of the C-terminal thiol(s), we have expanded the mechanism to explicitly include an EH_4–$NADP^+$–Hg(II) complex (C) with $FADH_2$ as the actual reductant of the multiliganded Hg(II), as proposed in the preceding paper by Distefano et al. (1989). Such an intermediate must exist only transiently, since no significant amount of reduced flavin is observed during either multiple-turnover (Sandstrom & Lindskog, 1988; Miller et al., unpublished observations) or single-turnover experiments conducted at pH 7.3 (Miller et al., unpublished observations). Movement of electrons from NADPH to FAD (B to C) should be facilitated by the liganding of the Cys_{140} thiolate to Hg(II) in species B,

Scheme III: Possible Mechanisms for Reduction of Multiliganded Hg(II) by Enzyme-Bound $FADH_2$[a]



[a] (A) Addition–elimination pathway. (B) Formation of a flavin C-4a thiol adduct concomitant with an outer-sphere reduction of Hg(II). (C) Successive single-electron transfers. It should be noted that the liganding of Hg(II) or Hg(I) and the protonation state of the thiol ligands in these mechanisms are uncertain. For simplicity, only one possibility is given.

since such liganding disrupts the thiolate to FAD charge-transfer interaction that is responsible for lowering the reduction potential for the FAD in EH_2 relative to that for FAD in E_{ox}.

The chemical mechanism by which $FADH_2$ reduces the liganded Hg(II) (conversion of C to D in Scheme II) is not yet established. However, we envision three reasonable mechanisms for this transformation, which are outlined in Scheme III. Mechanism A involves a nucleophilic attack of the flavin C-4a on the electropositive Hg(II), followed by elimination of Hg^0. Precedence for this mechanism lies in its similarity to the chemistry of the allylic oxidation of olefins (Rappoport et al., 1968, 1970; Fieser & Fieser, 1967) and to the mercuration reactions of ring systems similar to the rings of $FADH_2$ (Marshall et al., 1984; Dale et al., 1975). The one drawback of this postulate, however, is that we know of no examples in the literature of these reactions occurring in the presence of strong Hg(II) ligands such as thiols. Thiol complexes of Hg(II) are generally less reactive than the $Hg(OAc)_2$ and $Hg(NO_3)_2$ compounds used in the references above. Thus, it is not clear whether the addition–elimination pathway can occur on the enzyme where the Hg(II) ligands are known to be thiols.

Mechanism B in Scheme III involves a nucleophilic attack of $FADH_2$ on the Cys_{140} thiol, which is liganded to Hg(II). This results in an outer-sphere reduction of Hg(II) to Hg^0 concomitant with formation of an intermediate flavin C-4a thiol adduct. While there is no direct precedent in the chemical literature for such an outer-sphere reduction of Hg(II), such mechanisms, which occur by electron transfer via a bridging ligand, have been observed with other metals such as Cu(II) (Kochi, 1974) and Cr(II) (Taube, 1959). Arguments for this mechanism occurring in mercuric reductase derive from data on the analogous pyridine nucleotide oxidoreductases as well as from data on the reductive half-reaction of mercuric reductase itself. For glutathione reductase, which forms thiolate to FAD charge-transfer complexes with spectral properties essentially identical with those of mercuric reductase (Bulger & Brandt, 1971a,b), analysis of the crystal structure (Thieme et al., 1981) revealed that the charge-transfer thiolate lies above the plane of the flavin in the proper alignment for formation of a C-4a adduct. Such an adduct is believed to be an intermediate in the transfer of electrons between pyridine nucleotide and the active site disulfide in all of these homologous enzymes. Indeed, formation of a stable C-4a thiol adduct has been observed upon binding of NAD^+ to a modified form of lipoamide dehydrogenase, EHR, where complete transfer of electrons to NAD^+ cannot occur due to alkylation of the interchange thiol of the active site thiol pair (Thorpe & Williams, 1981). Furthermore, the C-4a thiol adduct appears to be the primary intermediate formed upon reduction of mercuric reductase with NADPH at low pH (Sahlman et al., 1986) and suggests that such an intermediate may be an important species in all of the redox chemistry of mercuric reductase.

Mechanism C in Scheme III involves two successive single-electron transfers from reduced flavin to Hg(II), with a Hg(I) complex as an intermediate. The best precedent for such a mechanism is the reported reduction of Hg(II) by flavin semiquinone (Singh et al., 1982). Singh et al. (1982b) have also shown in a comparative study with coordinately saturated Co(III) complexes that $FADH_2$ is even more reactive than flavin semiquinone in electron-transfer reactions. An electron-transfer mechanism may also be the best parallel to the reduction of Hg(II) in a $Hg(Cys)_2$ complex that occurs at the hanging drop mercury electrode (Stankovich & Bard, 1977).

To summarize, in this paper we report the presence of an auxiliary dithiol in mercuric reductase which is required for catalytic activity. We have shown the dithiol to be composed of the C-terminal thiols Cys_{558} and Cys_{559}. As a working hypothesis we propose that these thiols, along with the active site thiol pair, provide a polycoordinate binding site for Hg(II)

in the viable enzyme–substrate complex, which modulates its reduction potential to facilitate reduction by the enzyme-bound $FADH_2$. We are currently conducting studies to test this hypothesis and to further elucidate the chemical mechanism of Hg(II) reduction on the enzyme.

Added in Proof

By using ^{13}C NMR, Cheesman et al. (1988) have demonstrated recently that glutathione (GSH) and cysteine form trithiol complexes of Hg(II) [$Hg(SG)_3$] in a pH-dependent reaction. From their measured value of K_a for $Hg(SG)_3$, they estimate that, under physiological conditions (pH 7.4 and 2.2 mM glutathione), a solution 10 μM in total Hg(II) would contain nearly 11% as $Hg(SG)_3$. In view of the positioning of 4 thiol groups in the active site of mercuric reductase, which provide an effective high concentration of thiol ligands, these results strongly support our proposal that the auxiliary dithiol participates in catalysis to form at least a 3-coordinate Hg(II) complex.

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