# Directed Mutagenesis of the Redox-Active Disulfide in the Flavoenzyme Mercuric Ion Reductase†

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ABSTRACT: Mercuric ion reductase, a flavoenzyme with an active site redox-active cystine, $Cys_{135}$-$Cys_{140}$, is an unusual enzyme that reduces Hg(II) to Hg(0) with stoichiometric NADPH oxidation. To probe the catalytic mechanism, we have constructed two active site Cys to Ser mutations by oligonucleotide-directed mutagenesis. The native and the $Cys_{135}$, $Ser_{140}$ and $Ser_{135}$, $Cys_{140}$ mutant enzymes are expressed on an overproducing plasmid and purified to homogeneity by a one-step procedure in high yield. The optical spectra of the mutant proteins are distinct, with the $Ser_{135}$, $Cys_{140}$ mutant displaying a thiolate–flavin charge-transfer band ($Cys_{140}$ p$K_a$ = 5.2), confirming that $Cys_{140}$, not $Cys_{135}$, is in charge-transfer distance both in this mutant and in two electron reduced native enzyme. The native and both mutant proteins are dimers and are precipitated by antibody to native enzyme. Thiol titrations with 5,5′-dithiobis(2-nitrobenzoate) (DTNB) indicate that both mutants contain three kinetically accessible thiols in both oxidized and reduced states. The native enzyme has two titratable thiols when oxidized and four in the two electron reduced state. The native and two Cys to Ser mutant enzymes show differentiable NADPH-dependent catalytic behavior with $Hg(SR)_2$ (R = $CH_2CH_2OH$), $Hg(CN)_2$, DTNB, thio-$NADP^+$, and $O_2$, the most striking of which are the activities toward the Hg(II) complexes and DTNB. Only native enzyme reduces $Hg(SR)_2$. The $Ser_{135}$, $Cys_{140}$ enzyme alone shows sustained $Hg(CN)_2$ reduction, whereas the native and $Cys_{135}$, $Ser_{140}$ enzymes are rapidly inactivated. DTNB reduction is catalyzed by the native and $Cys_{135}$, $Ser_{140}$ enzymes, but not by the $Ser_{135}$, $Cys_{140}$ enzyme. The DTNB and $Hg(CN)_2$ reductions catalyzed by the distinct mutant enzymes suggest that monodentate chelation to a given enzyme SH may be sufficient for a given activity and that in these cases reduction occurs by direct electron transfer from $FADH_2$.

The flavoenzyme mercuric ion reductase has the unique ability to catalytically reduce Hg(II) to Hg(0):

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

This function coupled with subsequent volatilization of Hg(0) from the cell constitutes the molecular basis for bacterial resistance to mercuric ions (Summers & Silver, 1978; Robinson & Tuovinen, 1984). Recent structural studies of mercuric reductase (Fox & Walsh, 1982, 1983) and sequencing of the Tn501-encoded structural gene, mer A (Brown et al., 1983), reveal an active site redox-active disulfide and remarkable active site homology with the flavoprotein oxidoreductases, human glutathione reductase, and pig heart lipoamide dehydrogenase (Williams et al., 1982). In particular, the active site disulfide and the intervening four residues are completely conserved (Jones & Williams, 1975; Krohne-Ehrich et al., 1977; Matthews et al., 1974; Brown & Perham, 1972; Burleigh & Williams, 1972). Furthermore, two-electron reduction of all three enzymes results in the formation of an active site dithiolate that is capable of complexing mercuric ions (Massey & Williams, 1965; Casola & Massey, 1966). Mercuric ion reductase, however, is the only enzyme with the capability to catalytically reduce bound Hg(II) to Hg(0).

We have begun to address the mechanism of mercuric ion reduction by this unique enzyme via oligonucleotide-directed mutagenesis of the active site cystine disulfide residues. These residues, $Cys_{135}$ and $Cys_{140}$, have been shown to play a key role in catalysis (Fox & Walsh, 1982, 1983), serving as one of two two-electron acceptors in the active site (FAD is the second) and, in the reduced form, acting as the Hg(II) ion binding site. We describe here the catalytic and physical properties of two mercuric ion reductase mutants, the $Ser_{135}$, $Cys_{140}$ and $Cys_{135}$, $Ser_{140}$ mutants, in which $Cys_{135}$ and $Cys_{140}$, respectively, have been altered to serine residues. Both mutants show a variety of catalytic activities, including NADPH-dependent reduction of $Hg(CN)_2$.

## Materials and Methods

### *Materials*

*Escherichia coli* strain DS714 carrying the plasmid pJOE114 (Brown et al., 1983) was the generous gift of Dr. Nigel Brown of the Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K. *E. coli* strain W3110 $lacI^q$ and plasmid pSE181 were generously provided by Dr. Graham Walker of the Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 01239. *E. coli* strain JM101 and M13mp9 RF DNA were obtained from New England Biolabs.

All restriction enzymes, T4 polynucleotide kinase, T4 ligase, and DNA polymerase I (Klenow fragment) were obtained from New England Biolabs. Calf intestinal alkaline phosphatase was obtained from Boehringer-Mannheim. Reagents and enzymes for Sanger sequencing, deoxyadenosine 5′-[$\alpha$-$^{32}$P]triphosphate (>400 Ci/mmol), and adenosine 5′-[$\gamma$-$^{32}$P]triphosphate (>5000 Ci/mmol) were from Amersham. Deoxyribonucleotide triphosphates were from Boehringer-Mannheim, and riboATP was from Sigma.

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Protected dinucleotides for oligonucleotide synthesis were obtained from P-L Biochemicals. Substituted (aminomethyl)polystyrene resins were obtained from Vega.

Orange A Mātrex gel was purchased from Amicon Corp. Ampicillin sodium salt, IPTG,[1] TPCK, TLCK, thio-$NADP^+$, and Xgal were from Sigma. DTNB was purchased from Aldrich. Carba-1-deazariboflavin and 8-hydroxyriboflavin were generously provided by Merck Sharp & Dohme Research Laboratories, Rahway, NJ.

### *Methods*

*Spectrometry.* UV spectra were recorded on a Perkin-Elmer 554 or Lambda 5 spectrophotometer. Fluorescence spectra were recorded on a Perkin-Elmer LS-3 fluorometer.

*Oligonucleotide Synthesis.* Oligonucleotides were synthesized from protected 3′-terminating deoxydinucleotide phosphotriesters by the solid-phase phosphotriester method of Itakura (Tan et al., 1983). After removal of the phosphate and base protecting groups and cleavage from the (aminomethyl)polystyrene support, the 5′-DMT oligonucleotides were purified by reverse-phase chromatography with a 2 mL/min 20-min linear gradient from 15 to 30% $CH_3CN$ in 0.1 M triethylammonium acetate buffer (pH 7.2). The trityl group was then removed, and the resulting deoxyoligonucleotide was purified by reverse-phase chromatography with a 20-min linear gradient run at 2 mL/min from 5 to 30% $CH_3CN$ in 0.1 M triethylammonium acetate (pH 7.2). The oligonucleotide was then 5′-phosphorylated with 5′-[$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase and sequenced by a modified Maxam and Gilbert protocol (Zoller & Smith, 1983).

*Plasmid Construction.* (*A*) *M13ps1.* The 1748 base pair *Hin*dIII–*Sal*I fragment of pJ0E114 was isolated by low melting point agarose gel electrophoresis and ligated in the presence of *Hin*dIII–*Sal*I-cleaved M13mp9 (RF) DNA pretreated with calf intestinal alkaline phosphatase. The resulting mixture was used to transform competent *E. coli* JM101. Six colorless plaques were then selected from YT plates containing 3% Xgal and 0.6% IPTG. Single-strand and replicative-form DNA were isolated, and RF DNA was shown to contain the desired insert by analysis with the restriction enzymes *Hin*dIII, *Sal*I, and *Eco*RI.

(*B*) *pPS01.* The 3550 base pair *Nar*I–*Pvu*II pJ0E114 fragment and large dephosphorylated *Cla*I–*Sma*I pSE181 fragment were isolated by low melting point gel electrophoresis and ligated in the presence of T4 DNA ligase. The resulting mixture was used to transform *E. coli* W3110 lacI$^q$, and six Ap$^R$ colonies were selected on YT plates containing 50 $\mu$g/mL ampicillin. Plasmid isolated from each colony was in each case shown to contain the desired insert by restriction analysis with the enzyme *Eco*RI. Insertion of the 3550 base pair *Nar*I–*Pvu*II fragment of plasmids pPSM2 ($Ser_{135}$, $Cys_{140}$) and pPSM3 ($Cys_{135}$, $Ser_{140}$) into *Cla*I–*Sma*I-cleaved pSE181 afforded plasmids pPS02 and pPS03, respectively.

(*C*) *pPSM2 and pPSM3.* The 1748 base pair *Hin*dIII–*Sal*I fragment of M13ps2 or M13ps3 replicative-form DNA was ligated to the large dephosphorylated *Hin*dIII–*Sal*I fragment of pJOE114 by methods described above and the resulting plasmid DNA isolated. These procedures yielded the mutant merA-containing plasmids pPSM2 ($Ser_{135}$, $Cys_{140}$) and pPSM3 ($Cys_{135}$, $Ser_{140}$). The DNA encoding the active site residues (amino acids 130–180) was sequenced from the unique *Sal*I site of these plasmids by the method of Maxam & Gilbert (1979).

*Mutagenesis.* Oligonucleotide-directed mutagenesis was carried out by the method of Zoller & Smith (1983), with the exception of extending the mutagenesis primer in the presence of a universal sequencing primer (Messing et al., 1983), located 280 bases from the $Cys_{135}$ codon, and deleting the alkaline sucrose gradient centrifugation. Primer specificity was assayed by in vitro primer extension followed by *Hae*III and *Nci*I digestion and denaturing gel electrophoresis. Optimal specificity for both primers was obtained by annealing 30 pmol of primer to 1 pmol of M13ps1 template at 55 °C for 10 min, followed by cooling to 0 °C for 20 min and extension. For the mutagenesis reactions, extension and ligation were carried out under the above conditions for 18 h at 15 °C in the presence of 5 pmol of New England Biolab sequencing primer 1211. Mutants were screened by dot-blot hybridization with wash temperatures of 58 °C for the $Ser_{135}$, $Cys_{140}$ mutant (5′-ACATTGACGCTGGTGCC-3′, $T_D$ = 54 °C) and 26 °C for the $Cys_{135}$, $Ser_{140}$ mutant (5′-CAGCCTTCA-CACGGCAG-3′, $T_D$ = 56 °C). After plaque purification and a second round of hybridization screening, the presence of the desired mutation was confirmed by Sanger sequencing (Sanger et al., 1981). The resulting mutant plasmids are M13ps2 ($Ser_{135}$, $Cys_{140}$) and M13ps3 ($Cys_{135}$, $Ser_{140}$).

*Enzyme Purification.* The following procedure applies to native enzyme (pPS01) and the $Ser_{135}$, $Cys_{140}$ (pPS02) and $Cys_{135}$, $Ser_{140}$ (pPS03) mutant enzymes. During purification, Hg(II) reductase activity was assayed in the case of the native enzyme and transhydrogenase activity (thio-$NADP^+$/NADPH) for mutant enzymes. Assay procedures are described below.

Cells (W3110 lacI$^q$/pPS01, pPS02, pPS03) were grown in 8 L of LB media at 37 °C in a shaker bath. Native or mutant mercuric reductase was induced when $A_{550}$ of the fermentation broth = 1.0 by the addition of IPTG to a final concentration of 2 mM. Cells were harvested at a cell density of 220 Klett units (measured on a Klett–Summerson photoelectric colorimeter equipped with a red filter) by centrifugation for 10 min at 6000*g*. Typically, 40 g (wet weight) of cells was harvested (Table I).

The remainder of the purification follows the procedures of Fox & Walsh (1982) with the following modifications: (a) 0.01 mg/mL each of TPCK and TLCK was added to the purification buffer; (b) the heat-precipitation step preceding Orange A Mātrex Gel chromatography was omitted. The proteins appeared homogeneous by SDS gel electrophoresis and were used for experiments reported in this paper. Purified mercuric reductase (native and mutant) could be stored at 4 °C over a period of weeks with little or no loss of activity. For extended periods, samples (1–2 mg/mL) containing 10% w/v glycerol were frozen in liquid nitrogen and stored at −70 °C.

*Enzyme Assays.* Hg(II)-, thio-$NADP^+$-, and DTNB-dependent NADPH oxidations were monitored at 37, 25, and 25 °C, respectively, in 80 mM sodium phosphate, pH 7.4, and 200 $\mu$M NADPH. Hg(II)- and thio-$NADP^+$-dependent NADPH oxidations were monitored at 340 (absorbance) or 470 nm (emission). NADPH-dependent DTNB reduction was monitored at 440 nm ($\epsilon$ = 9.5 $mM^{-1}$ $cm^{-1}$). Hg(II) reductase activity was monitored in the presence of 100 $\mu$M $HgCl_2$ and 1 mM 2-mercaptoethanol or in the presence of 200 $\mu$M $Hg(CN)_2$ without thiols. Inactivation experiments with HgEDTA

[1] Abbreviations: IPTG, isopropyl $\beta$-D-thiogalactopyranoside; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; TLCK, $N^{\alpha}$-*p*-tosyl-L-lysine chloromethyl ketone; Xgal, 5-bromo-4-chloro-3-indolyl $\beta$-D-galactopyranoside; DTNB, 5,5′-dithiobis(2-nitrobenzoate); DMT, 4,4′-dimethoxytrityl; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

were carried out in the presence of 100 $\mu$M $HgCl_2$ and 0.5 mM EDTA. The high stability constant of HgEDTA ensures that all Hg(II) is present as the EDTA chelate (Sillen & Martell, 1964; Rinderle et al., 1983). Transhydrogenase activity was monitored in the presence of 1 mM 2-mercaptoethanol and 100 $\mu$M thio-$NADP^+$ during enzyme purifications. For $K_m$ and $V_{max}$ determinations, enzyme samples were dialyzed against 20 mM sodium phosphate, 0.5 mM EDTA, pH 7.4 buffer to remove $NADP^+$ introduced during Orange A column chromatography. DTNB reductase activity was monitored in the presence of 1 mM DTNB. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the Hg(II)- or thio-$NADP^+$-dependent oxidation of 1 $\mu$mol of NADPH/min or the reduction of 0.5 $\mu$mol of DTNB/min.

Oxygen consumption assays were performed at 37 °C on a Yellow Springs Instrument Co. biological oxygen monitor Model 53 with air-saturated buffer and 150 $\mu$M NADPH. Enzyme samples had been dialyzed to remove thiols. Production of $H_2O_2$ was confirmed by the addition of catalase to the reaction mixture after $O_2$ consumption began to level off.

*Protein Concentration.* Protein concentrations were determined by the method of Lowry (1951) with bovine serum albumin as a standard. Routine determination of enzyme concentration was based on flavin content, with an extinction coefficient of 11.3 $mM^{-1}$ $cm^{-1}$ at 458 nm for native enzyme, 11.3 $mM^{-1}$ $cm^{-1}$ at 440 nm for $Cys_{135}$, $Ser_{140}$ enzyme, and 9.48 $mM^{-1}$ $cm^{-1}$ at 440 nm for $Ser_{135}$, $Cys_{140}$ enzyme. These values were determined by first measuring the absorbance of a sample of the enzyme at the visible absorption $\lambda_{max}$. The flavin was then liberated by boiling for 15 min in 100 mM sodium phosphate (pH 7.4) and 10 mM $MgCl_2$. The precipitated protein was removed by centrifugation, and the free flavin absorbance at 450 nm was measured.

*Molecular Weight Determination.* Subunit molecular weight was determined by SDS–polyacrylamide gel electrophoresis as described by Laemmli (1970). Native molecular weight was determined by polyacrylamide gel electrophoresis. Samples for native gel electrophoresis were loaded in 50 mM Tris-HCl, pH 7.0, 0.1% 2-mercaptoethanol, 20% w/v glycerol, and 0.001% bromophenol blue.

*Quantitation and Removal of Enzyme-Bound $NADP^+$.* Enzyme-bound $NADP^+$ was quantitated or removed as described by Fox & Walsh (1982).

*Thermal Titrations.* Enzyme was heated in 80 mM sodium phosphate buffer containing 0.1 mM EDTA and 0.1% 2-mercaptoethanol for 10 min in air-tight polypropylene tubes. Samples were then cooled to 0 °C, centrifuged at 12000*g*, and assayed for transhydrogenase activity at 25 °C.

*Thiol Titrations.* Thiols were titrated with DTNB as described previously (Fox & Walsh, 1982).

*Antibody Preparation.* Rabbit antiserum against wild-type mercuric reductase was prepared from a New Zealand 2-kg white female rabbit. The rabbit was injected intramuscularly at 3-week intervals for 9 weeks with a 1:1 mixture of 1 mg/mL native mercuric reductase and 1 mL of Freund's complete adjuvant (Difco) in 20 mM sodium phosphate, pH 7.4. Serum was frozen in liquid nitrogen and stored at −70 °C until use. Routine precipitations were carried out by adding equal proportions of antisera and enzyme in 80 mM sodium phosphate, pH 7.4, incubating at 25 °C for 10 min, and centrifuging at 12000*g* for 5 min.

*Anaerobic and Redox Titrations.* Anaerobic and redox titrations were performed as previously described in this laboratory (Fox & Walsh, 1982) or in the laboratory of Prof. Vincent Massey, Department of Biological Chemistry, University of Michigan, Ann Arbor, MI by procedures to be presented in a later paper. To avoid formation of charge-transfer complexes with nicotinamides, $NADP^+$ was removed from all preparations of enzyme as previously described. The redox indicator dyes 1-deazariboflavin [$E^{\circ\prime}$ = −280 mV (Walsh et al., 1978)], 8-hydroxyriboflavin [$E^{\circ\prime}$ = −340 mV (Light & Walsh, 1980)], methyl viologen [$E^{\circ\prime}$ = −449 mV (Wilson, 1978)], and benzyl viologen [$E^{\circ\prime}$ = −358 mV (Wilson, 1978)] were used to determine oxidation–reduction potentials of the mutant mercuric reductases.

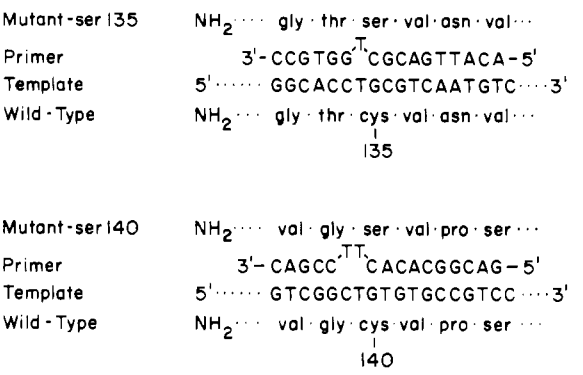


FIGURE 1: Primers used to generate the $Cys_{135}$, $Ser_{140}$ and $Ser_{135}$, $Cys_{140}$ mutant mercuric reductases.

## RESULTS

*Generation and Expression of Mercuric Reductase Mutants.* Mutagenesis was carried out on a 1748 base pair active site containing merA fragment (encoding amino acids 1–186 of the enzyme) subcloned from the plasmid pJ0E114 into the single-stranded phage M13mp9. The method of Zoller & Smith (1983) was used with modifications described under Methods. The mutagenesis primers 5′-ACATT-GACGCTGGTGCC-3′ and 5′-GACGGCACACTTCC-GAC-3′ contain one mismatch at the seventh base [$Cys_{135}$ (TGC) → Ser (AGC)] and two mismatches at the sixth and seventh bases [$Gly_{139}$ (GGC) → Gly (GGA); $Cys_{140}$ (TGT) → Ser (AGT)], respectively (Figure 1). Overall mutagenesis yields of 3% were obtained for each primer.

After verification of the desired mutation by Sanger sequencing with a universal sequencing primer, the 1748 base pair mutant *Hin*dIII–*Sal*I M13ps1 fragment was subcloned into pJ0E114 to reconstruct the merA gene. The resulting plasmids pPSM2 ($Cys_{135}$ → Ser) and pPSM3 ($Cys_{140}$ → Ser) were sequenced by the method of Maxam & Gilbert (1979) to verify insertion of the active site containing mutant sequence.

Plasmid pJ0E114 was expressed in the host *E. coli* JM101 and induced by incubating cells in 10–20 $\mu$M merbromin. Purification by methods previously described (Fox & Walsh, 1982) yielded 125 units (25 mg) of enzyme from 40 g of cells. Mutant enzymes (pPSM2 and pPSM3) could not be purified by this procedure. To increase expression and facilitate purification, we placed merA under control of the hybrid tac promoter (de Boer et al., 1982) contained in the plasmid pSE181. The 3550 base pair *Nar*I–*Pvu*II merA-containing fragments of pJ0E114, pPSM2, and pPSM3 were cloned into *Cla*I- and *Sma*I-digested pSE181 (Figure 2). The resulting plasmids pPS01, pPS02, and pPS03 were expressed in *E. coli* W3110 $lacI^q$ and induced with the gratuitous inducer IPTG. The crude soluble cellular extract contained 5% mercuric reductase (pPS01) as determined by Hg(II) reductase activity, an approximate 6-fold enhancement.

The purification procedure of Fox & Walsh has been modified to a one-step (Orange A Mātrex dye column) procedure applicable to both native and mutant enzymes (Table

Table I: Purification of Native and Mutant Mercuric Reductases

| enzyme | total protein (mg) | sp act. (units/mg) | tot act. (units) | x-fold purification | yield (%) |
|---|---|---|---|---|---|
| native[a] | | | | | |
| crude | 4015 | 0.031 | 126 | | |
| Orange A | 142 | 0.96 | 137 | 30.7 | 108 |
| native[b] | | | | | |
| crude | 4015 | 0.24 | 944 | | |
| Orange A | 142 | 6.4 | 909 | 30.5 | 96 |
| $Ser_{135}$, $Cys_{140}$[a] | | | | | |
| crude | 3600 | 0.0044 | 15.8 | | |
| Orange A | 176 | 0.100 | 17.6 | 22.7 | 111 |
| $Cys_{135}$, $Ser_{140}$[a] | | | | | |
| crude | 3300 | 0.066 | 219 | | |
| Orange A | 146 | 1.42 | 207 | 21.4 | 95 |

[a] Enzyme activity was determined by the thio-$NADP^+$/NADPH transhydrogenase assay as described under Methods. [b] Enzyme activity was determined by the mercuric ion reductase assay as described under Methods.

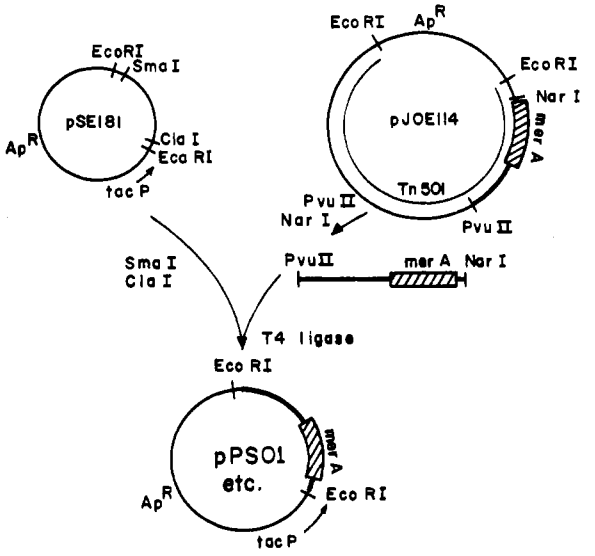


FIGURE 2: Overproducer construction for native and mutant mercuric reductase.

I). Up to 150 mg of enzyme can be purified on a 2.5 × 20 cm Orange A column. Since both the $Ser_{135}$, $Cys_{140}$ and $Cys_{135}$, $Ser_{140}$ mutant enzymes are qualitatively more susceptible to proteolysis during purification, the addition of the protease inhibitors TPCK and TLCK to the crude lysate is required.

*Thiol Titrations.* Thiols were titrated in the reduced and oxidized forms of the native and mutant enzymes with DTNB. Enzymes were denatured prior to titration with 5 M guanidine hydrochloride. The presence in wild-type enzyme of two titratable thiols in the oxidized form and four titratable thiols upon reduction by NADPH (Fox & Walsh, 1982) confirmed here as 2.2 and 4.3 thiols per subunit, respectively, is consistent with the presence of a redox active disulfide. Both the $Cys_{135}$, $Ser_{140}$ and $Ser_{135}$, $Cys_{140}$ mutants contain three titratable thiols in both the oxidized and reduced forms (3.1 and 3.0 for $Cys_{135}$, $Ser_{140}$ enzyme and 3.2 and 3.2 for $Ser_{135}$, $Cys_{140}$ enzyme). This result is consistent with the substitution of one cysteine by serine in each mutant. In the case of oxidized nondenatured $Cys_{135}$, $Ser_{140}$ mutant, three thiols can be titrated with DTNB, indicative of aryl disulfide–thiol exchange in the active site. Only two of the three thiols could be titrated in nondenatured $Ser_{135}$, $Cys_{140}$ mutant enzyme.

*Physical Properties.* The $Cys_{135}$, $Ser_{140}$ and $Ser_{135}$, $Cys_{140}$ mutants share many physical properties with wild-type enzyme. Both mutant and native enzymes have dimeric structures with subunit $M_r$ of 62 000. The protein to flavin ratio, $A_{272}/A_{458}$ for native enzyme and $A_{272}/A_{440}$ for the mutants, is 6.2–6.3. When FAD content is compared with protein concentration as determined by the method of Lowry, the stoichiometry of FAD binding is one FAD per subunit.

$NADP^+$ is associated with the pure native and mutant enzymes, even after extensive dialysis. For native enzyme, the stoichiometry is 1 $NADP^+$ per 2.7 FAD (Fox & Walsh, 1982). For both the $Cys_{135}$, $Ser_{140}$ and $Ser_{135}$, $Cys_{140}$ mutants, the stoichiometry is 1 $NADP^+$ per 3.2 FAD.

The thermal stabilities of the three enzymes are comparable. The midpoint of the heat-inactivation curve for the $Cys_{135}$, $Ser_{140}$ mutant is 76 °C, for the $Ser_{135}$, $Cys_{140}$ mutant 78.5 °C, and for oxidized native enzyme 83 °C. The disulfide bridge has relatively little effect on the thermal stability of the enzyme toward heat denaturation, not too surprisingly since the cystine disulfide bridges only four amino acid residues.

Antibodies raised against native enzyme cross-react with both mutants. All three enzymes lost greater than 90% of the transhydrogenase activity when titrated with antiserum. In the case of highly homologous glutathione reductase, evidence points to an antigenic determinant at the nicotinamide binding domain of the enzyme (Carlberg et al., 1981).

*Spectroscopic Properties.* Native mercuric reductase can exist in an oxidized state (E), a two electron reduced state ($EH_2$), and a four electron reduced state ($EH_4$), each of which have distinctive electronic absorption spectra (Figure 3A). The oxidized state is characterized by a $\lambda_{max}$ in the visible region at 458 nm (Fox & Walsh, 1982). $EH_2$ contains oxidized flavin and reduced disulfide ($Cys_{135}$ and $Cys_{140}$) at the active site. This change is reflected in a 28% decrease in the 458-nm absorbance in the $NADP^+$-free enzyme, a shift of $\lambda_{max}$ to 440 nm, and a new absorbance band at 540 nm. This long-wavelength absorbance is ascribed to a charge-transfer complex between one of the thiolate anions in the active site and oxidized flavin (Fox & Walsh, 1982; Thorpe & Williams, 1981). Further addition of electrons results in the loss of absorbance in both the long wavelength absorbing chromophore and the flavin, consistent with net four electron reduction of the disulfide and the flavin.

Both the oxidized $Ser_{135}$, $Cys_{140}$ and $Cys_{135}$, $Ser_{140}$ enzymes lack the redox-active disulfide and consequently have only a two electron reduction capacity. The spectrum of the oxidized $Ser_{135}$, $Cys_{140}$ enzyme (Figure 3B), with a $\lambda_{max}$ at 440 nm ($\epsilon$ = 9.48 $mM^{-1}$ $cm^{-1}$) and long-wavelength absorption at 540 nm, resembles that of $EH_2$ in native enzyme (Figure 3A). The presence of the 540-nm band in this mutant clearly demonstrates the involvement of $Cys_{140}$ in the thiolate–flavin charge-transfer complex. The absorbance spectra of native $EH_2$ and oxidized $Ser_{135}$, $Cys_{140}$ enzyme differ in the charge-transfer region, possibly through the influence of the residue at position 135 on the charge-transfer complex. Re-

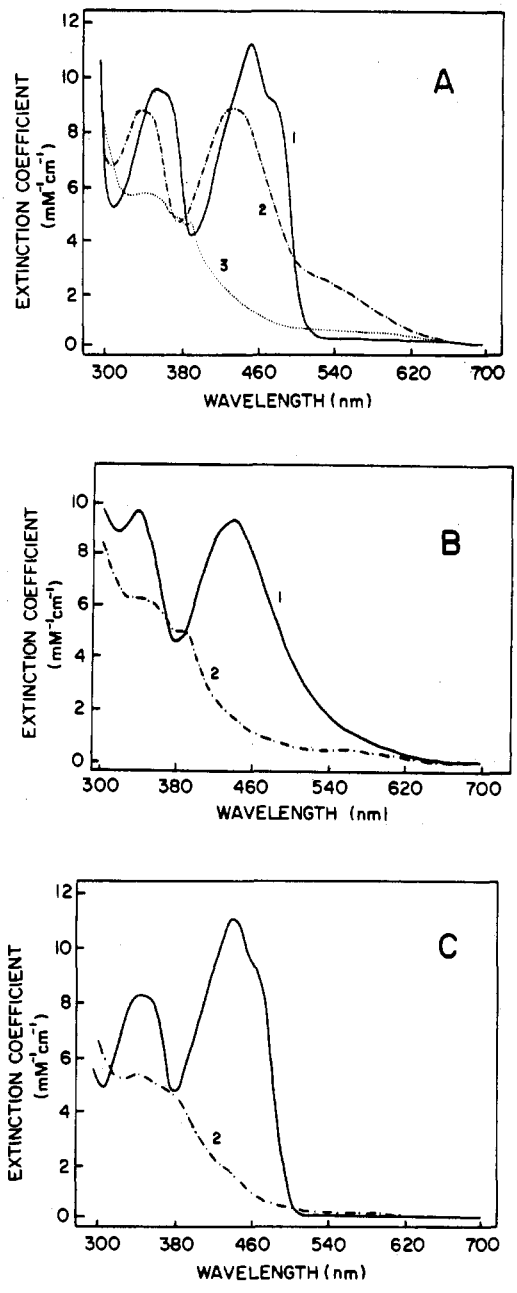


FIGURE 3: Optical spectra of oxidized and reduced native and mutant mercuric reductases. In (A–C), spectrum 1 is oxidized enzyme, spectrum 2 is two electron reduced enzyme, and spectrum 3 is four electron-reduced enzyme. (A) Native enzyme; (B) $Ser_{135}$, $Cys_{140}$ enzyme; (C) $Cys_{135}$, $Ser_{140}$ enzyme.

duction of oxidized $Ser_{135}$, $Cys_{140}$ enzyme by two electrons results in the loss of the charge-transfer and flavin band, consistent with formation of $FADH_2$.

The electronic absorption spectrum of oxidized $Cys_{135}$, $Ser_{140}$ mutant mercuric reductase, which has $\lambda_{max}$ at 440 nm ($\epsilon$ = 11.3 $mM^{-1}$ $cm^{-1}$), resembles that of oxidized native enzyme (Figure 3C). The shift in $\lambda_{max}$ to higher energy in the mutant vs. native enzyme may indicate partial stabilization of the flavin by the hydroxyl group at residue 140. Dithionite reduction of the oxidized $Cys_{135}$, $Ser_{140}$ enzyme again results in the loss of the 440-nm absorbance, indicative of $FADH_2$ formation. The lack of a charge-transfer complex in this mutant suggests that $Cys_{135}$ is not in close proximity to the bridgehead C-4a position of the flavin.

For native enzyme, the fluorescence emission maximum of the native enzyme at 30 °C is at 520 nm and the excitation maximum is at 483 nm (Fox & Walsh, 1982). The fluores-

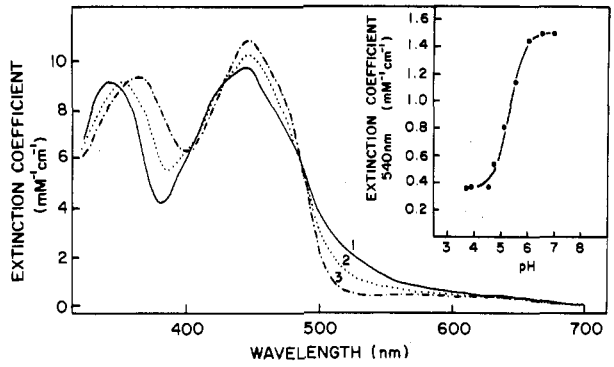


FIGURE 4: Determination that $Cys_{140}$-SH $pK_a$ = 5.2. Three representative spectra showing the decrease of absorbance at 540 nm with decreasing pH are shown.

cence of the native enzyme is 3.1 times more intense than that of free FAD at the same concentration. The fluorescence emission and excitation maxima at 20 °C of the $Ser_{135}$, $Cys_{140}$ mutant enzyme are at 518 and 475 nm, respectively, with an intensity one-third that of free FAD. The emission and excitation maxima for the $Cys_{135}$, $Ser_{140}$ enzyme at 20 °C are at 508 and 468 nm, respectively (intensity 1.7 times that for free FAD).

*$Cys_{140}$ $pK_a$ Determination.* The $Ser_{135}$, $Cys_{140}$ mutant enables us to determine the $pK_a$ of the thiolate anion involved in the charge-transfer complex by monitoring the extinction coefficient of the 540-nm absorbance as a function of pH. As the pH is lowered, the spectrum of the oxidized enzyme approaches that of free FAD (Figure 4). These absorption changes are compatible with protonation of the anionic electron donor, with consequent loss in the charge-transfer band. The titration curve is reversible down to pH 3.0 and affords a $pK_a$ for $Cys_{140}$ of 5.2. For comparison, the $pK_a$ of the corresponding cysteine in pig heart lipoamide dehydrogenase is 5.2 (Matthews & Williams, 1976) and 4.8 for glutathione reductase (Arscott et al., 1981). Relatively little difference is seen in the absorption spectrum of the $Cys_{135}$, $Ser_{140}$ mutant from pH 6.0 to pH 12, and there is no optical indication of titration of an acid–base group.

*Oxidation–Reduction Potential Determination.* The oxidation–reduction potentials of the two mutant enzymes were determined by dithionite titrations in 80 mM sodium phosphate (pH 7.4) in the presence of the redox indicator dyes 1-deazaflavin and benzyl viologen ($Cys_{135}$, $Ser_{140}$) and 8-hydroxyriboflavin and methyl viologen ($Ser_{135}$, $Cys_{140}$). The midpoint potential in native enzyme of the $E/EH_2$ couple is −269 and −335 mV for the $EH_2/EH_4$ couple (Fox & Walsh, 1982). The midpoint potential for the $E/EH_2$ couples in the two mutants are clearly more negative than that in the native enzyme. Determinations of potential are complicated by the possibility that the flavins on the two subunits display cooperativity (V. Massey, personal communication). Preliminary indications are that the $Ser_{135}$, $Cys_{140}$ mutant potential is probably below −375 mV, while the $Cys_{135}$, $Ser_{140}$ mutant potential may be in the −350-mV range (V. Massey, personal communication). Full quantitative redox analyses will be presented in a subsequent paper.

*Enzymatic Activity.* We have assayed five NADPH-dependent activities in both the native and mutant mercuric reductases: $Hg(SR)_2$ and $Hg(CN)_2$ reduction, aryl disulfide reduction, transhydrogenation (thio-$NADP^+$/NADPH), and $O_2$ reduction (Table II).

All mercuric reductase enzymes isolated to date show a requirement of exogenous thiols for Hg(II) reduction (Robinson & Tuovinen, 1984). For example, native mercuric re-

Table II: Catalytic Activities of Native and Mutant Mercuric Reductase[a]

| assay | native enzyme | $Cys_{135}$, $Ser_{140}$ | $Ser_{135}$, $Cys_{140}$ |
|---|---|---|---|
| $Hg(SR)_2$ reduction | 740 (2nd phase)[b] | none (<3) | none (<3) |
| $Hg(CN)_2$ reduction | killed | killed | 13 |
| DTNB reduction | 440 | 13 | none |
| transhydrogenation | 46 | 56 | 20 |
| $O_2$ reduction | 4 | 17 | 0.4 |

[a] Activities are expressed as turnover numbers per dimer per minute. [b] Fox & Walsh, 1982.

ductase (Tn501) shows Hg(II) reductase activity in the presence of exogenous 1 mM 2-mercaptoethanol (Fox & Walsh, 1982). The kinetics of NADPH oxidation for the Tn501-encoded mercuric reductase have been shown to be biphasic with $V_{max}$ (first phase) of 800 $min^{-1}$ per FAD and $K_m$ for $HgCl_2$ of 12 $\mu$M (Fox & Walsh, 1982). Enzyme dialyzed to remove enzyme-bound $NADP^+$ showed a lower $V_{max}$, 310 $min^{-1}$ per FAD. Native enzyme is rapidly inactivated by HgEDTA ($t_{1/2}$ = 20 s) or $Hg(CN)_2$ ($t_{1/2}$ = 80 s) in the absence of thiol reagents, as indicated by the decrease in the rate of NADPH oxidation (data not shown). Incubation of the $EH_2$ form of native enzyme, generated by anaerobic dithionite titration, with 100 $\mu$M $Hg(CN)_2$ in the absence of NADPH results in the disappearance of the charge-transfer band at 540 nm. This observation is compatible with complexation of $Cys_{140}$ thiolate anion in $EH_2$ with Hg(II), either as a monodentate $Cys_{140}$-S–Hg–CN or bidentate $Cys_{140}$-S–Hg–S-$Cys_{135}$ complex. Subsequent incubation with thiols reactivates the HgEDTA- or $Hg(CN)_2$-inactivated enzyme complex. Similar results have been reported previously by Rinderle et al. (1983) on the pRR130-encoded mercuric reductase.

The $Ser_{135}$, $Cys_{140}$ and $Cys_{135}$, $Ser_{140}$ mutant mercuric reductases show no reductase activity toward Hg(II) in the presence of 1 mM 2-mercaptoethanol (turnover number < 3 $min^{-1}$). However, on screening a number of $HgX_2$ salts, it was found that the $Ser_{135}$, $Cys_{140}$ mutant catalytically reduces $Hg(CN)_2$ in the presence of NADPH. The kinetics in this case are not biphasic but follow Michaelis–Menten behavior. The $V_{max}$ is 13 $min^{-1}$, and $K_m$ for $Hg(CN)_2$ is 48 $\mu$M. Since the $Ser_{135}$, $Cys_{140}$ mutant has only a two-electron redox capacity, catalytic Hg(II) reduction must be occurring via direct electron transfer from FAD. Addition of 100 $\mu$M $Hg(CN)_2$ to the oxidized $Ser_{135}$, $Cys_{140}$ enzyme in the absence of NADPH results in disappearance of the charge-transfer band seen in oxidized enzyme. As was the case with native enzyme, the loss of the charge-transfer band suggests the formation of a monodentate $Cys_{140}$-S–Hg–CN or bidentate $Cys_{140}$-S–Hg–O-$Ser_{135}$ adduct.

For the $Cys_{135}$, $Ser_{140}$ mutant, incubation with 100 $\mu$M $Hg(CN)_2$ leads to rapid inactivation ($t_{1/2}$ = 80 s) of the mutant enzyme, rather than sustained Hg(II) reductive turnover.

The native and the two mutant mercuric reductases show no detectable (<3 $min^{-1}$) NADPH oxidation activity in the presence of the disulfides glutathione and lipoamide. However, native mercuric reductase does reduce the aryl disulfide 5,5′-dithiobis(2-nitrobenzoate) (DTNB) in the presence of NADPH with a $V_{max}$ of 440 $min^{-1}$ and $K_m$ for DTNB of 400 $\mu$M, in contrast to the pRR130-encoded Hg(II) reductase, which has been recently reported to have a $K_m$ of 3 mM for DTNB and $V_{max}$ of 10 $min^{-1}$, 1% of the Hg(II) reduction rate (Rinderle et al., 1983).

The $Cys_{135}$, $Ser_{140}$ mutant also reduces DTNB in the presence of NADPH with a $V_{max}$ of 13 $min^{-1}$ and $K_m$ for DTNB of 500 $\mu$M, close to the $K_m$ of 400 $\mu$M in native enzyme. In the absence of NADPH, the $Cys_{135}$, $Ser_{140}$ mutant releases a stoichiometric amount of 2-nitrobenzoate thiolate anion, strongly suggesting DTNB disulfide exchange with $Cys_{135}$ leading to the formation of the $Cys_{135}$-S–S-nitrobenzoate mixed disulfide. The $Ser_{135}$, $Cys_{140}$ mutant shows no NADPH oxidation activity in the presence of 1 mM DTNB, nor is the active site thiol titratable in nondenatured enzyme.

The native and mutant enzymes all show transhydrogenase activity (thioNADP$^+$/NADPH) as do glutathione reductase and lipoamide dehydrogenase. For the native enzyme, the $V_{max}$ is 46 $min^{-1}$, and $K_m$ for NADPH is 0.8 $\mu$M; for the $Cys_{135}$, $Ser_{140}$ mutant, $V_{max}$ is 56 $min^{-1}$, and $K_m$ is 0.8 $\mu$M; for the $Ser_{135}$, $Cys_{140}$ mutant, $V_{max}$ is 20 $min^{-1}$, and $K_m$ is 3 $\mu$M. The transhydrogenase activity of the mutants is not too surprising, since the iodoacetamide $Cys_{135}$-alkylated native enzyme has a 2.5-fold higher $V_{max}$ for transhydrogenation than native enzyme (Fox & Walsh, 1983). Transhydrogenase activity in the mutant and alkylated enzymes does not correlate with Hg(II) reductase activity, consistent with independent nicotinamide and Hg(II) binding domains. Since these enzymes have only a two-electron capacity and only one nicotinamide binding site, transhydrogenation is likely to result from intermediate reduction and reoxidation of FAD.

The native and mutant mercuric reductases all show $O_2$-dependent NADPH oxidation activity. Native enzyme has a $k_{cat}$ of 4 $min^{-1}$, $Ser_{135}$, $Cys_{140}$ enzyme a $k_{cat}$ of 0.4 $min^{-1}$, and $Cys_{135}$, $Ser_{140}$ enzyme a $k_{cat}$ of 17 $min^{-1}$ in air-saturated buffer at 37 °C.

## Discussion

In order to understand the unique structural properties of mercuric reductase that make possible Hg(II) reduction, we have begun to construct active site mutants and characterize their physical and catalytic properties. Although no X-ray crystal structure is yet available for mercuric reductase (crystallization attempts are under way), the enzyme's primary structure bears strong homology to that of human red cell glutathione reductase. There is an excellent 1.9-Å resolution map available for glutathione reductase alone and in its complex with $NADP^+$ or glutathione (Thieme et al., 1981; Schulz et al., 1982; Pai & Schulz, 1983). The structural similarity between the two enzymes, especially the identity of 12 residues in the active site tryptic peptide, suggested that the glutathione reductase structure would serve as an initial framework against which to plan mutant mercuric reductase species. The glutathione reductase X-ray structure coupled with previous physical studies of mercuric reductase strongly point to the active site cysteines as playing a key role in Hg(II) binding and reduction. We have therefore constructed two active site mutants, the $Cys_{135}$, $Ser_{140}$ and $Ser_{135}$, $Cys_{140}$ mutants, in which $Cys_{140}$ and $Cys_{135}$, respectively, have been altered to serine residues.

Native mercuric reductase in addition to glutathione reductase and lipoamide dehydrogenase is isolated with both FAD and the active site cysteines oxidized as the intramolecular disulfide. This is termed the oxidized (E) form of the enzyme. Two-electron reduction of oxidized enzyme generates $EH_2$, which, when first formed, is likely to contain $FADH_2$, on the basis of the X-ray structure of glutathione reductase in which FAD is sandwiched between $NADP^+$ and $Cys_{58}$-S–S-$Cys_{63}$. The electrons, however, are rapidly transferred from $FADH_2$ to the active site disulfide, and the equilibrium form of $EH_2$ is characterized by a charge-transfer complex between one of the cysteine thiols and FAD.

Iodoacetamide treatment of the active site cysteines in $EH_2$

affords an 18/1 regioselective labeling of $Cys_{135}$ vs. $Cys_{140}$ (Fox & Walsh, 1983), a selectivity for the amino proximal cysteine of the dithiol pair mirroring that seen with glutathione reductase (Arscott et al., 1981). Since the alkylated mercuric reductase displayed a charge-transfer band, provisionally assigned to $Cys_{140}$ and oxidized FAD, we anticipated that of the two mutants, $Ser_{135}$, $Cys_{140}$ and $Cys_{135}$, $Ser_{140}$, only the former would show a charge-transfer band. This is in fact the case and is consistent with the glutathione X-ray structure in which the carboxy proximal cysteine is in close proximity to bound FAD.

The presence of the charge-transfer band in the spectrum of the $Ser_{135}$, $Cys_{140}$ mutant permitted the facile titration of the $Cys_{140}$ thiol $pK_a$. The value of 5.2 reflects a 3 orders of magnitude stabilization of the thiolate in its active site geometry compared with a cysteine free in solution. The thiolate stabilization is due at least in part to the stabilization provided by interaction with FAD in the charge-transfer complex and may also indicate the involvement of an active site base (Williams, 1976; Matthews & Williams, 1976; Untucht-Grau et al., 1979). Furthermore, the reduction potential for the $FAD/FADH_2$ couple in the $Ser_{135}$, $Cys_{140}$ mutant (−375 to −400 mV) is substantially more negative than that of the $EH_2/EH_4$ couple in the native enzyme.

On addition of two additional electrons to the $EH_2$ form of native mercuric reductase, the four-electron capacity of the enzyme is saturated and $E–FADH_2$ accumulates. This change is reflected in the disappearance of the 454-nm FAD absorption in the four electron reduced enzyme, $EH_4$ (Figure 3A). It is worth explicit note that the mutation of either $Cys_{135}$ or $Cys_{140}$ to serine produces mutant mercuric reductases that in their fully oxidized state, E, are functionally equivalent to the $EH_2$ form of the native enzyme. These mutants contain oxidized FAD and an active site thiol. Correspondingly, the two electron reduced mutant enzymes, with $FADH_2$ in the active site, are functionally equivalent to the $EH_4$ form of native enzyme, the only form of native enzyme with a significant content of $FADH_2$. Consequently, the electronic absorption spectrum of both $EH_2$ mutant enzymes resembles that of native $EH_4$ (Figure 3). The active site Cys to Ser mutations have converted mercuric reductase into a catalyst with only a two-electron redox inventory rather than a four-electron redox inventory. This fact coupled with the different loci of the remaining active site cysteine residue is a useful content for analysis of the catalytic properties of the $Cys_{135}$, $Ser_{140}$ and $Ser_{135}$, $Cys_{140}$ mutant mercuric reductases.

We have assayed five catalytic activities in this work: (a) transhydrogenation from NADPH to thio-$NADP^+$, (b) reduction of the aryl disulfide 5,5′-dithiobis(2-nitrobenzoic acid), (c) reduction of $O_2$ to $H_2O_2$, (d) reduction of mercuric dimercaptide complexes, and (e) reduction of mercuric dicyanide (Table II). All five redox reactions involve the bound FAD, but it is likely that only reactions b, d, and e require participation of either active site cysteine.

We previously found that mercuric reductase, monoalkylated at cysteine 135 and therefore incompetent for Hg(II) reduction (Fox & Walsh, 1983), still catalyzed hydride transfer between NADPH and thio-$NADP^+$. The $V_{max}$ for this activity was up 2-fold in alkylated enzyme. These results were consistent with the active site geometry found in glutathione reductase in which NADPH and the cystine disulfide lie on opposite faces of the bound FAD (Pai & Schulz, 1983). Thus, we anticipated that the $Cys_{135}$, $Ser_{140}$ and $Ser_{135}$, $Cys_{140}$ mutants would be competent in the transhydrogenase half-reaction (Figure 5). The nicotinamide–flavin half-reaction, as assayed

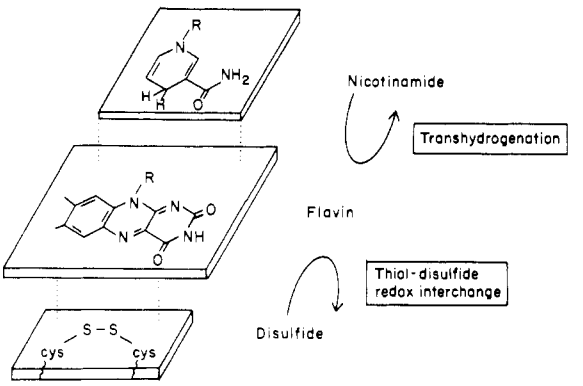


FIGURE 5: A model for the mercuric reductase active site—the active site geometry of glutathione reductase. The NADPH and cystine disulfide are located on opposite faces of the bound FAD (Pai & Schulz, 1983).

by transhydrogenation, does in fact survive both Cys to Ser mutations, with the $Cys_{135}$, $Ser_{140}$ mutant having a $V_{max}$ 20% higher than that in native enzyme. The 2–3-fold differences in $K_m$ and $V_{max}$ between the native and two mutant enzymes are not interpretable at present.

Although rapid reduction of $O_2$ to $H_2O_2$ is a property of dihydroflavins free in solution, in many flavoproteins reaction of bound dihydroflavin with $O_2$ is strongly suppressed. This is the case with native mercuric reductase where $O_2$ reduction at 4 $min^{-1}$ is only 1% the $k_{cat}$ for Hg(II) reduction. The low oxidase activity in the native enzyme may result from the fact that the predominant catalytic species in Hg(II) reduction is $EH_2$, which contains FAD, not $FADH_2$, and so would be nonreactive toward $O_2$. If Hg(II) reduction does involve the $EH_4$ form of the enzyme in turnover, then $FADH_2$ would be stoichiometrically present and low oxidase activity may result from steric constraints to $O_2$ approach. In the $Cys_{135}$, $Ser_{140}$ mutant, the $O_2$ reduction activity is up 4-fold to 17 $min^{-1}$. In the case of this mutant, turnover must generate $FADH_2$ in each catalytic cycle, there being no other electron sink available.

The remaining three activities, aryl disulfide, $Hg(CN)_2$, and $Hg(SR)_2$ reduction, focus more specifically on the role of $Cys_{135}$ and $Cys_{140}$ in mercuric ion reduction by this unique enzyme. Reduction of the chromogenic aryl disulfide 5,5′-dithiobis(2-nitrobenzoate) is rapid in the native enzyme, with a $V_{max}$ of 440 $min^{-1}$. The $Cys_{135}$, $Ser_{140}$ mutant can also reduce DTNB catalytically with a $V_{max}$ of 13 $min^{-1}$, 3% the rate of native. The lack of detectable catalytic activity of the $Ser_{135}$, $Cys_{140}$ mutant toward DTNB (<3 $min^{-1}$) sharply differentiates the function of the two active site cysteines. The detection of catalytic DTNB reduction in the $Cys_{135}$, $Ser_{140}$ mutant strongly suggests that, in the absence of the $Cys_{140}$ thiolate, $FADH_2$ is directly supplying reducing equivalents to the mixed $Cys_{135}$-S–S-aryl disulfide to produce ArS– and oxidized mutant enzyme. The diminished rate of DTNB reduction by the $Cys_{135}$, $Ser_{140}$ mutant relative to native mercuric reductase may reflect either altered FAD redox properties, altered binding geometries in the active site, or a change in mechanism for DTNB reduction. There remains the question of why the $Ser_{135}$, $Cys_{140}$ mutant shows no detectable DTNB reduction activity. Recall that iodoacetamide shows an 18/1 preference for alkylation of $Cys_{135}$ over $Cys_{140}$ (Fox & Walsh, 1983). If this preference reflects steric accessibility, the relatively bulky DTNB may not be able to reach the $Cys_{140}$ thiolate to form a mixed $Cys_{140}$-S–S-aryl disulfide. This point can be probed by testing a small disulfide with a redox potential close to that of DTNB.

This discussion brings us to the main mechanistic question: can either mutant, each of which is limited to monodentate thiol ligation and a two-electron capacity, reduce mercuric ions? Under standard assay conditions with dimercaptide Hg(II) salts, neither enzyme shows any detectable activity (<3 $min^{-1}$). However, with $Hg(CN)_2$, the $Ser_{135}$, $Cys_{140}$ mutant catalyzes reductive turnover at a rate of 13 $min^{-1}$, which proceeds without detectable inhibition. On the other hand, both the native and $Cys_{135}$, $Ser_{140}$ mercuric reductases catalyze $Hg(CN)_2$ turnover but are rapidly inactivated.

The reduction of $Hg(CN)_2$ by the $Ser_{135}$, $Cys_{140}$ mutant clearly indicates that Hg(II) can be reduced at the active site by monodentate thiol complexation. [We are in the process of testing whether $Ser_{135}$ or $Ser_{140}$ is involved in Hg(II) chelation by constructing Cys to Ala mutants.] In particular, addition of $Hg(CN)_2$ to this mutant in the absence of NADPH dissipates the charge-transfer band, indicative of Hg(II) chelation to the $Cys_{140}$ thiolate. Although not conclusive, these observations suggest that native mercuric reductase may bind and reduce Hg(II) as a monodentate complex with $Cys_{135}$ or $Cys_{140}$.

A second key point is that reduction of Hg(II) to Hg(0) by both mutants must result from direct electron transfer from $FADH_2$, the only reductant in the two mutant enzymes. The net two-electron transfer from $FADH_2$ to $Cys_{140}$-S–HgX could involve any of the three following processes: two one-electron transfers involving flavin semiquinone and Hg(I) as intermediates, direct hydride transfer from N-5 of $FADH_2$, or attack of $FADH_2$ to form a $Cys_{140}$–4a-flavin adduct.

There remains then the questions of why $Hg(SR)_2$ is not reduced by the $Ser_{135}$, $Cys_{140}$ mutant enzyme (or by the $Cys_{135}$, $Ser_{140}$ mutant) and why the rate with $Hg(CN)_2$ by the $Ser_{135}$, $Cys_{140}$ mutant is reduced relative to that of $Hg(SR)_2$ by native enzyme. The first observation may reflect differences in substrate redox potentials, and studies with other metal ion complexes may be useful in exploring this possibility. However, we do not yet understand the properties that allow native enzyme and not the Cys to Ser mutants to catalytically reduce $Hg(SR)_2$ salts, nor do we understand the reason for the lower rate of turnover of mercuric salts by the mutant enzyme.

The autoinactivation of the native and $Cys_{135}$, $Ser_{140}$ enzymes by $Hg(CN)_2$ is reminiscent of HgEDTA inactivation of the pRR130 mercuric reductase reported by Rinderle et al. (1983). The premise there and here is that the enzyme may have a bidentate $Cys_{135}$-S–Hg–S-$Cys_{140}$ complex at the active site, the same kind of bidentate thiol chelation that characteristically inhibits other enzymes. This proposal is supported by the fact that subsequent addition of RSH to the inactivated enzyme restores catalytic activity, presumably by thiol ligand exchange to regenerate the active monodentate thiol–Hg(II) complex. What is then the mechanism for turnover-linked inactivation of the $Cys_{135}$, $Ser_{140}$ mercuric reductase? The $pK_a$ of $Ser_{140}$ in this enzyme is as yet unknown but could be substantially lower than that of free serine in solution due to its proximity to the 4a position of FAD (3 $pK_a$ units lower for Cys). The hydroxyl could therefore be sufficiently acidic to form an inactive bidentate $Ser_{140}$-O–Hg–S-$Cys_{135}$ complex. In the $Ser_{135}$, $Cys_{140}$ mutant, the $Ser_{135}$ is not in electronic contact with the FAD, would not have a substantially altered $pK_a$, and may not be capable of forming an inactive $Ser_{135}$-O–Hg–S-$Cys_{140}$ complex. This proposition is being tested by construction of a $Cys_{135}$, $Ala_{140}$ mutant mercuric reductase to see whether it gives sustained turnover of $Hg(CN)_2$.

In summary, the construction of the active site cysteine to serine mutants and the rapid isolation of large amounts of pure enzyme have begun to allow us to unravel the mechanism by which this unique enzyme can reduce Hg(II) salts, a process of fundamental importance in molecular toxicology, inorganic metabolism, and environmental geochemistry.

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## REFERENCES

Arscott, L. D., Thorpe, C., & Williams, C. H. (1981) *Biochemistry 20*, 1513.
Brown, J. P., & Perham, R. N. (1972) *FEBS Lett. 26*, 221.
Brown, N. L., Ford, S. J., Pridmore, R. D., & Fritzinger, D. C. (1983) *Biochemistry 22*, 4089.
Burleigh, B. D., & Williams, C. H. (1972) *J. Biol. Chem. 247*, 2077.
Carlberg, I., Altmejd, B., & Mannervik, B. (1981) *Biochim. Biophys. Acta 677*, 146.
Casola, L., & Massey, V. (1966) *J. Biol. Chem. 241*, 4985.
de Boer, H. A., Comstock, L. J., Yansura, D. G., & Heynecker, H. L. (1982) in *Promoters, Structure and Function* (Rodriguez, R. L., & Chamberlin, M. J., Eds.) pp 462–481, Praeger, New York.
Fox, B. S., & Walsh, C. T. (1982) *J. Biol. Chem. 257*, 2498.
Fox, B. S., & Walsh, C. T. (1983) *Biochemistry 22*, 4082.
Jones, E. T., & Williams, C. H. (1975) *J. Biol. Chem. 250*, 3779.
Krohne-Ehrich, G., Schirmer, R. H., & Untucht-Grau, R. (1977) *Eur. J. Biochem. 80*, 65.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Light, D. R., & Walsh, C. (1980) *J. Biol. Chem. 255*, 4264.
Loechler, E. L., & Hollocher, T. C. (1980) *J. Am. Chem. Soc. 102*, 7322.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Massey, V., & Williams, C. H. (1965) *J. Biol. Chem. 240*, 4470.
Matthews, R. G., & Williams, C. H. (1976) *J. Biol. Chem. 251*, 3956.
Matthews, R. G., Arscott, L. D., & Williams, C. H. (1974) *Biochim. Biophys. Acta 370*, 26.
Maxam, A., & Gilbert, W. (1979) *Methods Enzymol. 65*, 499.
Messing, J., Crea, R., & Seeburg, P. H. (1981) *Nucleic Acids Res. 9*, 309.
Pai, E. F., & Schulz, G. E. (1983) *J. Biol. Chem. 258*, 1752.
Rinderle, S. J., Booth, J. E., & Williams, J. W. (1983) *Biochemistry 22*, 869.
Robinson, J. B., & Tuovinen, O. H. (1984) *Microbiol. Rev. 48*, 95.
Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. H., & Roe, B. A. (1981) *J. Mol. Biol. 143*, 161.
Schulz, G. E., Schirmer, R. H., & Pai, E. F. (1982) *J. Mol. Biol. 160*, 287.
Sillen, L. G., & Martell, A. E. (1964) *Spec. Publ.—Chem. Soc. 17*, 637.
Summers, A. O., & Silver, S. (1978) *Annu. Rev. Microbiol. 32*, 637.
Tan, Z. K., Ikuta, S., Huang, T., Dugaiczyk, A., & Itakura, K. (1983) *Cold Spring Harbor Symp. Quant. Biol. 47*, 383.
Thieme, R., Pai, E. F., Schirmer, R. H., & Schulz, G. E. (1981) *J. Mol. Biol. 152*, 763.
Thorpe, C., & Williams, C. H. (1981) *Biochemistry 20*, 1507.

Untucht-Grau, R., Schulz, G. E., & Schirmer, R. H. (1979) *FEBS Lett. 105*, 244.
Walsh, C., Fisher, J., Spencer, R., Graham, D. W., Ashton, W. T., Brown, J. E., Brown, R. D., & Rogers, E. F. (1978) *Biochemistry 17*, 1942.
Williams, C. H. (1976) *Enzymes (3rd Ed.) 13*, 89-173.
Williams, C. H., Arscott, L. D., & Schulz, G. E. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2199.
Wilson, G. S. (1978) *Methods Enzymol. 54*, 396.
Yokoe, I., & Bruice, T. C. (1975) *J. Am. Chem. Soc. 97*, 450.
Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 468.

# Protein Carboxyl Methylation-Demethylation System in Developing Rat Livers†

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ABSTRACT: Protein carboxyl methyltransferase and protein methylesterase activity was assayed in various cell fractions prepared from rat livers. Significant amounts of protein carboxyl methyltransferase were detected in the cytosol and nucleoplasm. The cellular concentration of this enzyme paralleled development, activity being highest in the liver from young animals. If methylation was inhibited at any point during the reaction with *S*-adenosylhomocysteine, protein methylesterase activity was evident by a rapid decrease in carboxyl-methylated proteins. Protein methylesterase activity could be assessed by measuring the amount of [$^3$H]methanol present in reaction filtrates. After a 10-min lag, the rate of demethylation was equivalent to the rate of methylation. The turnover of methyl groups was primarily enzymatic, since little or no methanol was generated when adrenocorticotropic hormone was incubated with purified protein carboxyl methyltransferase. Assessment of protein methylesterase activity as a function of the amount of methanol in the reaction filtrates represents minimal values, since the resultant [$^3$H]methanol was metabolized rapidly via an alcohol dehydrogenase and/or oxidase. The rapid turnover of the protein methyl esters makes it difficult to assess the endogenous methyl acceptor proteins. Protein methyl esters were not detectable in any significant amounts in hepatic cell fractions in vivo; however, the nuclei contained measurable amounts of carboxyl-methylated proteins in vitro. These proteins are firmly bound to DNA but are not an integral part of the nucleosome. Analysis of the proteins, after fractionation on hydroxylapatite and sodium dodecyl sulfate-acrylamide gel electrophoresis, revealed that several non-histone chromosomal proteins were carboxyl methylated. The approximate molecular weights of these proteins were 172K, 106K, 98K, 81K, 66K, 62K, 52K, and 38K. Results of binding studies suggest that these proteins originate in the nucleoplasm, perhaps after synthesis in the cytoplasm.

Protein carboxyl methyltransferase activity has been found in various rat tissues (Axelrod & Daly, 1965; Liss & Edelstein, 1967). This enzyme catalyzes the transfer of methyl groups from *S*-adenosylmethionine to the free carboxyl group of glutamyl and/or aspartyl residues in various proteins to form methyl esters (Kim & Paik, 1970). The protein methyl esters have been reported to be quite labile, undergoing hydrolysis to form methanol under physiological conditions (Axelrod & Daly, 1965; Kim & Paik, 1970). However, the lability of protein methyl esters is most likely due to the presence of a protein methylesterase. Gagnon (1979) has reported the presence of a heat-labile methylesterase in various rat tissues which readily hydrolyzes protein methyl esters, yielding methanol.

In bacteria, the carboxyl methylation-demethylation system has been found to play a role in chemotaxis (Black et al., 1982). This system involves the transfer of methyl groups from *S*-adenosylmethionine to specific methyl acceptor proteins in the bacterial cell membrane. Subsequent hydrolysis of the glutamyl methyl esters, via a methylesterase, results in the regeneration of the methyl acceptor protein and methanol (Stock & Koshland, 1978).

The function of the protein carboxyl methyltransferase-protein methylesterase system has not been delineated in eucaryotes. Diliberto & Axelrod (1976) have suggested that such a system may play a role in neurosecretion since high levels of protein carboxyl methyltransferase activity were found throughout the brain. The high concentrations of the enzyme in nonneuronal tissues (Duerre et al., 1985; O'Dea et al., 1981) would suggest that the enzyme system functions in a more general role and may regulate one or more processes in eucaryotes. One proposed function closely related to bacterial chemotaxis is leukocyte migration. The movement of leukocytes in response to a chemoattractant was found to enhance protein carboxyl methylation (O'Dea et al., 1978; Venkatasubramanian et al., 1980). Furthermore, the chemotactic response could be blocked with the addition of methylation inhibitors (Cantoni et al., 1979). van Waarde (1982) and van Waarde & van Haastert (1984) have demonstrated an increase in carboxyl methylation during aggregation of *Dictyostelium discoideum* amoeba in the presence of cAMP. Most of the methyl groups were incorporated within 15 s and lost within 1 min. The transient nature of the methylation-demethylation of proteins in *D. discoideum* is comparable to that observed

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