# Mercuric Reductase: Homology to Glutathione Reductase and Lipoamide Dehydrogenase. Iodoacetamide Alkylation and Sequence of the Active Site Peptide†

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ABSTRACT: The flavoprotein mercuric reductase encoded on the transposon Tn501 from *Pseudomonas aeruginosa* has previously been shown to contain a redox-active cystine at the active site and to share many spectrophotometric, physical, and kinetic properties with the nicotinamide disulfide oxidoreductases [Fox, B., & Walsh, C. T. (1982) *J. Biol. Chem. 257*, 2498–2503]. Oxidized mercuric reductase was unreactive toward iodo[$^{14}$C]acetamide, yet the two-electron-reduced form, in which the thiols of the redox-active cystine are free, reacted to give a monoalkylated derivative. The major $^{14}$C-labeled peptide from a tryptic digestion of labeled mercuric reductase was purified by high-performance liquid chromatography. The partial amino acid sequence of this peptide is Gly-Thr-Ile-Gly-Gly-Thr-SCMC-Val-Asx-Val-Gly-SCMC-Val-Pro. There is extensive sequence homology between this peptide and the active site peptides of lipoamide dehydrogenase and glutathione reductase. The specific activity of the labeled *S*-(carboxymethyl)cysteine residues indicated that the cysteine closer to the $NH_2$ terminus was 18-fold more reactive toward iodoacetamide than was the other cysteine. This suggests that, as with the nicotinamide disulfide oxidoreductases, the cysteine closer to the $NH_2$ terminus functions in substrate binding and is more accessible to solvent and iodoacetamide. The monoalkylated derivative of mercuric reductase could not catalyze $Hg^{2+}$-dependent reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidation but retained nicotinamide transhydrogenase activity. Alkylation changed the spectrum of mercuric reductase, generating a new long-wavelength absorbance band centered around 580 nm. It appeared that the charge-transfer complex between the cysteine closer to the COOH terminus and the oxidized flavin was intact but had been perturbed by the modification of the other cysteine. Mercuric reductase is also shown to specifically utilize the 4*S* hydrogen of NADPH during turnover. The amino acid sequences of the $NH_2$ termini of native and selectively proteolyzed mercuric reductase are presented, along with a discussion of the homology between mercuric reductase and the nicotinamide disulfide oxidoreductases.

The flavoprotein mercuric reductase (reduced NADP: mercuric ion oxidoreductase) contains two active site electron acceptors—FAD[1] and a redox-active disulfide (Fox & Walsh, 1982). This combination of redox centers is found in a few other flavoproteins as well, including glutathione reductase (EC 1.6.4.2) and lipoamide dehydrogenase (EC 1.6.4.3) (Williams, 1976). These latter two enzymes possess extensive sequence homology (Williams et al., 1982), most notably in the active site region, where the amino acids surrounding the redox-active cysteine residues are highly conserved (Jones & Williams, 1975; Krohne-Ehrich et al., 1977; Matthews et al., 1974; Brown & Perham, 1972; Burleigh & Williams, 1972). Mercuric reductase has recently been shown to share many spectrophotometric, physical, and kinetic properties with glutathione reductase and lipoamide dehydrogenase (Fox & Walsh, 1982).

These similarities are of particular interest due to the diverse biological functions of these enzymes. Glutathione reductase catalyzes the transfer of electrons from reduced pyridine nucleotides to exogenous disulfides, while lipoamide dehydrogenase preferentially catalyzes the reverse reaction. In the mercuric reductase reaction, however, electrons are transferred not to a cosubstrate disulfide but to inorganic mercuric ions. The utility of this reaction for the organism is clear—mercuric reductase plays a critical role in bacterial resistance to organomercurials and mercuric salts. The elemental mercury that is produced is volatile and is nonenzymatically removed from the growth medium. Mercury resistance is plasmid encoded and frequently present on a transposon (Summers & Silver, 1978; Stanisch et al., 1977; Summers et al., 1980), contributing to the high frequency of occurence of bacterial mercury resistance.

This paper describes studies on the sequence and conformation of the active site of mercuric reductase from *Pseudomonas aeruginosa* PAO9501 (pVS1). In this system, the *mer* operon is found on the transposon Tn501 (Stanisch et al., 1977), and mercuric reductase is the product of the *mer*A gene. The active site of the enzyme has been labeled with iodo-[$^{14}$C]acetamide, a reagent that has previously been shown to preferentially alkylate one of the redox-active cysteine residues of glutathione reductase (Arscott et al., 1981) and lipoamide dehydrogenase (Thorpe & Williams, 1976a, 1981). The iodoacetamide labeling has made possible the identification of the active site peptide and was also useful in the study of active site conformation. Sequencing of this modified peptide of mercuric reductase has directly addressed the extent of homology between mercuric reductase and the nicotinamide disulfide oxidoreductases. The *mer*A gene sequence of mercuric reductase from Tn501, which is described in the accompanying paper (Brown et al., 1983), has facilitated both the design of

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[1] Abbreviations: FAD, flavin adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; E, $EH_2$, and $EH_4$, oxidized, two-electron-reduced, and four-electron-reduced forms of mercuric reductase or of the nicotinamide disulfide oxidoreductases, respectively; EHR, monoalkylated derivative of these enzymes; thio-NADP$^+$, thionicotinamide adenine dinucleotide phosphate; AAD$^+$, 3-aminopyridine adenine dinucleotide; TPCK, $N^{\alpha}$-tosylphenylalanine chloromethyl ketone; HPLC, high-performance liquid chromatography; SCMC, *S*-(carboxymethyl)cysteine; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane. The amino acid derivatives resulting from Edman degradations are designated as PTH- (phenylthiohydantoin) and PTC- (phenylthiocarbamyl) amino acids.

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these experiments and the interpretation of the results.

## Experimental Procedures

*Materials.* Mercuric reductase from *Pseudomonas aeruginosa* PAO9501 (pVS1) was purified as reported previously (Fox & Walsh, 1982). The enzyme from this source is encoded on the transposon Tn501. Iodo[1-$^{14}$C]acetamide and [1-$^{3}$H]glucose were from New England Nuclear, chymotrypsin and TPCK-treated trypsin were from Worthington Biochemicals, and thio-NADP$^+$ was from P-L Biochemicals. All other reagents were of the highest grade commercially available.

*Enzyme Assays.* Routine enzyme assays, following $Hg^{2+}$-dependent NADPH oxidation, were performed as described previously (Fox & Walsh, 1982). Transhydrogenase activity (NADPH/thio-NADP$^+$) was monitored by following NADPH oxidation at 340 nm (close to the thio-NADPH/thio-NADP$^+$ isosbestic point at 338 nm). The assay mixtures contained 80 mM sodium phosphate, pH 7.4, 1 mM 2-mercaptoethanol, 200 $\mu$M NADPH, and 100 $\mu$M thio-NADP$^+$ at 37 °C. The turnover numbers of native mercuric reductase used in these studies were approximately 225 $min^{-1}$ for mercuric reductase activity and 90 $min^{-1}$ for transhydrogenase activity.

*UV–Visible Spectra.* Anaerobic titrations were performed in anaerobic cuvettes (Williams et al., 1979). Additions were made through a side arm with an attached gas-tight repeating syringe. Spectra were recorded at 25 °C on a Perkin-Elmer Model 554 spectrophotometer. To avoid the formation of charge-transfer complexes with nicotinamides, NADP$^+$ was removed from all preparations of mercuric reductase as described previously (Fox & Walsh, 1982).

*Partial Proteolysis.* Mercuric reductase was incubated at 0.5 mg/mL in 100 mM sodium phosphate, pH 7.4–0.5 mM EDTA with varying concentrations of chymotrypsin or trypsin for 30 min at room temperature. Proteolysis was monitored with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli, 1970). A preparative reaction utilized 16 mg of mercuric reductase and 64 $\mu$g of chymotrypsin (0.2 $\mu$g/mL) in 32 mL of the above buffer. After 30 min, the enzyme was separated from the protease by chromatography on a 50-mL Orange A Matrex gel column as described previously (Fox & Walsh, 1982). Protein prepared in this way will be referred to as clipped mercuric reductase.

*Determination of Stereochemistry of NADPH Oxidation.* (4*R*)-[4-$^{3}$H]NADPH was the generous gift of Dr. C. Ryerson of this laboratory and was prepared by using previously reported procedures (Ryerson et al., 1982). (4*S*)-[4-$^{3}$H]-NADPH was prepared enzymatically from [1-$^{3}$H]glucose and NADP$^+$ by using ATP, hexokinase, and glucose-6-phosphate dehydrogenase (Bergemeyer et al., 1974) and purified by ion-exchange chromatography (Ryerson et al., 1982). The radiolabeled NADPH was assayed with mercuric reductase as described above. The labilization of counts from [$^{3}$H]-NADPH was monitored by passing aliquots of the reaction mixture over a Dowex 1-$Cl^-$ column and by eluting with 2 × 1 mL of $H_2O$. The stereochemical purity of the (4*S*)-[4-$^{3}$H]NADPH was determined enzymatically. Glutathione reductase, specific for the 4*S* hydrogen of NADPH (Stern & Vennesland, 1960), labilized 63% of the counts upon prolonged incubation. This low percentage of labile counts was not due to racemization of the [$^{3}$H]NADPH, as cyclohexanone monooxygenase (the generous gift of Dr. B. Branchaud of this laboratory), specific for the 4*R* hydrogen of NADPH (Ryerson, 1980), labilized only 4% of the counts. By assuming that only 63% of the counts in solution were associated with active NADPH, the specific activity of the NADPH was calculated to be 20.9 $\mu$Ci/$\mu$mol.

*Iodo[$^{14}$C]acetamide Labeling of Mercuric Reductase.* The procedure used for iodoacetamide labeling is a modification of that described for pig heart lipoamide dehydrogenase (Thorpe & Williams, 1976a). The two-electron-reduced form of mercuric reductase, $EH_2$, was generated by the addition of sodium dithionite to an anaerobic solution containing 78.8 nmol of enzyme in 2.5 mL of 100 mM sodium phosphate, pH 7.4. The reduction was followed spectrophotometrically to ensure full formation of $EH_2$. Iodo[1-$^{14}$C]acetamide (2.3 mCi/mmol, 375 nmol in 0.15 mL of 100 mM sodium phosphate, pH 7.4) was added to the enzyme, and the reaction was allowed to proceed for 2.5 h at room temperature in the dark. The reaction was quenched with 1.75 $\mu$mol of L-cysteine in 35 $\mu$L of water. Similar conditions were used for smaller scale pilot studies.

The kinetics of labeling and inactivation were followed by quenching 0.1-mL aliquots of the reaction mixture into 0.05 mL of 50 mM L-cysteine. The residual enzymatic activity was assayed as described above. The stoichiometry of labeling was determined at each time point by measuring the associated radioactivity. The enzyme was precipitated with trichloroacetic acid (final concentration 10%, w/v), washed twice with acetone, and counted in Aqueous Counting Scintillant (Amersham) in the wide $^{14}$C channel of a Beckman LS-100 liquid scintillation counter. The concentration of enzyme was expressed in terms of the concentration of flavin present in the original solution (Fox & Walsh, 1982).

*Exhaustive Reduction and Alkylation.* The labeled mercuric reductase from the preparative labeling described above (78.8 nmol) was precipitated with trichloroacetic acid (final concentration 10%, w/v) and washed with acetone. The enzyme was then resuspended in 2.5 mL of 6 M guanidine hydrochloride, 300 mM Tris, pH 8.0, and 3 mM EDTA. Complete reduction was accomplished by the addition of 23 $\mu$mol of dithiothreitol and incubation at 37 °C for 1 h under argon. An excess of cold iodoacetamide (100 $\mu$mol) was then added to react with all remaining free thiols, followed by a 1-h incubation in the dark at room temperature. The reaction was quenched with 0.1 mL of 2-mercaptoethanol. Guanidine hydrochloride and other reagents were removed by extensive dialysis against four changes of 500 mL of 50 mM $NH_4HCO_3$. The resulting protein precipitate was resuspended in 2 mL of 88% HCOOH and then dialyzed against two changes of 1000 mL of $H_2O$. The protein was lyophilized in aliquots and stored at −20 °C.

*Proteolytic Digests of Alkylated Enzyme.* Alkylated mercuric reductase (5–15 nmol) was suspended in 0.25 mL of 10 mM HCOOH. TPCK-treated trypsin was added in 50 $\mu$L of 500 mM $NH_4HCO_3$, pH 8.1, to give a final trypsin concentration of 10 $\mu$g/mL. The proteolysis was quenched after 22 h at 25 °C with 25 $\mu$L of glacial acetic acid, and this reaction mixture was analyzed by HPLC.

*Purification of Radiolabeled Peptides by HPLC.* $^{14}$C-Labeled peptides were purified by reverse-phase chromatography on either a Waters Associates HPLC equipped with a Model 660 solvent programmer and a Model 441 absorbance detector or a Du Pont Instruments HPLC with a Series 8800 gradient controller and a Micromeritics 786 variable-wavelength detector. In either case, a Waters Associates $\mu$Bondapak analytical $C_{18}$ 10-$\mu$m column was used. A 70-min linear gradient was run at 2 mL/min from 7.5 to 47.5% $CH_3CN$ in aqueous 0.1% trifluoroacetic acid. Peptides were detected directly by their absorbance at 214 nm. Two-milliliter fractions were collected and aliquots counted as described above. Radioactive

Table I: $NH_2$-Terminal Sequence of Native Mercuric Reductase[a]

| PTH-amino acid | Thr | Thr | Leu | Lys | Ile | Thr | Gly | Met | Thr | X | Asp |
|---|---|---|---|---|---|---|---|---|---|---|---|
| nmol | b | 11[c] | 12 | 12 | 8 | 2 | 5 | 5 | 3 | | 3 |

[a] The sequence was determined twice, using samples from separate purifications. Quantitation of the number of nanomoles of PTH-amino acid at each residue is shown for one run, where approximately 15 nmol of proten had been applied to the sequenator. The values are approximate, as PTH-Nle was not added as an internal standard. [b] Quantitation of this first cycle was not possible due to the presence of contaminants. [c] The gene sequence indicates the presence of His at this position; His would not have been detected in our system. The PTH-Thr observed in this protein sequence may have been due to overlap from the Thr in the preceding position.

fractions were lyophilized and resuspended in 10 mM HCOOH. The major peak was pooled and rechromatographed with 12% $CH_3CN$ in aqueous 0.1% trifluoroacetic acid at 2 mL/min. The minor peak was rechromatographed by using a 40-min gradient from 7.5 to 17.5% $CH_3CN$ in aqueous 0.1% trifluoroacetic acid. Materials from different tryptic digests were handled separately.

*Protein Chemistry.* Amino acid compositions were obtained on a Dionex D-500 instrument after hydrolysis for 24 h in 0.1 mL of 6 N HCl. Amino acid sequence analysis was performed on a Beckman 890C sequenator maintained by the laboratory of Dr. R. T. Sauer, Department of Biology, Massachusetts Institute of Technology, using a double-cleavage 0.1 M Quadrol program (Brauer et al., 1975). For peptides, the sequenator cup was treated with 6 mg of polybrene (Tarr et al., 1978) prior to sample application. Sequence analysis was performed on 3–4 nmol of the $^{14}C$-labeled peptide with PTH-Nle added to each fraction as an internal standard. $NH_2$-terminal sequencing of intact and clipped mercuric reductase was performed with 15 and 10 nmol of purified protein, respectively. Conversions were carried out in 1 N HCl for 10 min at 80 °C (Ilse & Edman, 1963), and PTH derivatives were extracted with ethyl acetate. PTH-amino acids were identified on a Hewlett-Packard 5830A gas chromatograph using a 10% SP-400 column and on a Waters System HPLC with a Waters RCM-C18 column using a gradient program (Sauer et al., 1981), which could resolve all of the PTH-amino acids except PTH-Arg and PTH-His. PTH-Asp and PTH-Asn could be resolved with the program used for the $NH_2$-terminal sequence analyses but not with the slightly modified program used for the active site sequence determination.

## Results

*$NH_2$-Terminal Sequence Determination.* The $NH_2$-terminal sequence of mercuric reductase was analyzed by using enzyme samples prepared for sequence analysis within 24 h of the start of enzyme purification. This was necessary in order to minimize the problems of proteolytic degradation previously encountered (Fox & Walsh, 1982). The sequence obtained (Table I) is generally in agreement with that predicted by the *mer*A gene sequence (Brown et al., 1983); this $NH_2$-terminated sequence was provided to the Bristol group to enable them to localize the *mer*A gene for nucleotide sequencing. Some processing of the enzyme has clearly occurred, as the $NH_2$-terminal methionine, specified in the gene sequence, is absent from the isolated protein. Attempts to determine the $NH_2$-terminal sequence of mercuric reductase in the degraded form were unsuccessful. Multiple residues were obtained at each step of Edman degradation, supporting the previous hypothesis of $NH_2$-terminal degradation.

*Partial Proteolysis of Mercuric Reductase.* Partial proteolysis of mercuric reductase with chymotrypsin or TPCK-trypsin resulted in the formation of several discrete species with molecular weights of approximately 56 000 (Figure 1). Large-scale proteolysis with chymotrypsin was performed as described under Experimental Procedures. The $NH_2$-terminal sequence of this clipped mercuric reductase was determined (Table II) to locate the clip in the amino acid sequence. Comparison with the DNA sequence (Brown et al., 1983) reveals that 85 amino acids have been cleanly removed from the amino terminus of mercuric reductase. This processing had no apparent effect on the catalytic activity of the enzyme and did not alter its dimeric structure as determined by gel filtration (data not shown).

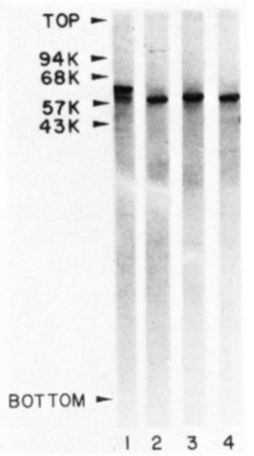



FIGURE 1: Partial proteolysis of mercuric reductase. Mercuric reductase was partially digested with proteases as described under Experimental Procedures. Approximately 2.5 μg of protein was loaded and electrophoresed on a 12% acrylamide gel. (Lane 1) Native mercuric reductase; (lane 2) enzyme plus chymotrypsin, 2 μg/mL; (lane 3) enzyme plus trypsin, 0.4 μg/mL; (lane 4) enzyme plus trypsin, 5 μg/mL. Molecular weights of standard proteins were as indicated.

Table II: $NH_2$-Terminal Sequence of Clipped Mercuric Reductase[a]

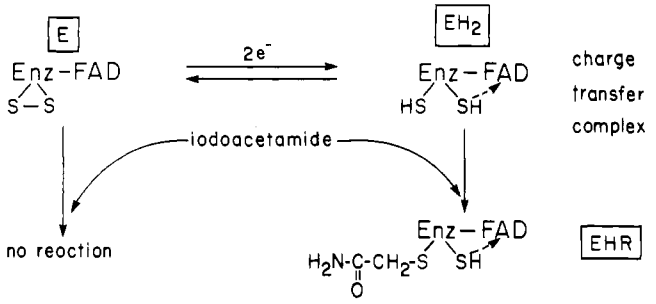
| PTH-amino acid | Met | Ala | Ala | Ala | Glu | Lys | X | X | Gly | Asn |
|---|---|---|---|---|---|---|---|---|---|---|
| nmol | 5.4 | 8.4 | 6.6 | 8.2 | 5.1 | 6.0 | | | 4.0 | 3.4 |

[a] Clipped mercuric reductase was prepared as described under Experimental Procedures. Approximately 10 nmol of protein was sequenced with a repetitive yield of 94%.

*Stereochemistry of NADPH Oxidation.* The stereospecificity of nicotinamide oxidation is a highly conserved characteristic of a given class of oxidoreductases and has been correlated with the orientation of binding of the pyridine nucleotide to the enzyme (You et al., 1978). Incubations of mercuric reductase with (4*R*)-[4-$^3H$]NADPH under normal assay conditions did not result in the release of any radioactivity over background levels. Incubations with (4*S*)-[4-$^3H$]NADPH, however, washed out 62% ($n$ = 6, SD = 6.2%) of the counts in solution, or 98% of the counts associated with active NADPH (i.e., those counts that could be released by 4*S*-specific glutathione reductase). Mercuric reductase, therefore, clearly utilizes the 4*S* hydrogen of NADPH. This stereochemistry of nicotinamide oxidation is the same as that exhibited by the nicotinamide disulfide oxidoreductases, indicating that the nicotinamides are bound in the same relative orientation.

*Properties of Alkylated Mercuric Reductase.* Mercuric reductase, when reduced to $EH_2$ and labeled with iodo[$^{14}C$]-acetamide, incorporated 1.1 ● 0.3 labels per FAD. The stoichiometric incorporation of radiolabel suggests that only one of the two active site cysteine residues exposed by the reduction is susceptible to alkylation. The monoalkylated

Scheme I




enzyme is termed EHR, by analogy to the alkylated form of pig heart lipoamide dehydrogenase, where it has been demonstrated that alkylation is associated with the loss of a proton (Thorpe & Williams, 1981). In a typical preparation of EHR, 95–99% of the $Hg^{2+}$-dependent NADPH oxidase activity was lost, although EHR retained the ability to transfer electrons from NADPH to thio-$NADP^+$. In fact, upon alkylation, transhydrogenase activity increased 2.5–6-fold. The final level of transhydrogenase activity rose to within 10% of the mercuric reductase activity of native enzyme (225 $min^{-1}$). Attempts to alkylate oxidized mercuric reductase with iodoacetamide under identical conditions resulted in essentially no labeling or inactivation. Very little incorporation of radioactivity was seen (0.06–0.09 label per FAD), and the catalytic behavior was unaffected. No more than 10% of the mercuric reductase activity was lost, and no increase in transhydrogenase activity was observed. Mercuric reductase is thus sensitive to alkylation by iodoacetamide only after two-electron reduction (Scheme I).

The kinetics of mercuric reductase alkylation appear to be biphasic. The spectral changes induced by alkylation (see below), as well as the increase in transhydrogenase activity, were essentially complete within 1 min. The loss of $Hg^{2+}$-dependent NADPH oxidase activity and the incorporation of radioactivity to a final stoichiometric level occurred at a slower rate, however. Both of these latter processes appeared to be 50% complete with 1 min.

The stoichiometric modification of mercuric reductase with iodoacetamide resulted in the formation of a novel spectral species. The spectrum is compared in Figure 2 with those of E, $EH_2$, and $EH_4$. The EHR spectrum did not change upon addition of $K_3Fe(CN)_6$ or upon extensive dialysis. Complete reduction with dithionite resulted in the formation of a spectrum typical of reduced flavin, and upon reoxidation with air, the 580-nm band reappeared. The long-wavelength absorbance band due to the charge-transfer complex in $EH_2$ (see Scheme I) is still present, although it is clearly modified.

*Purification of the Labeled Peptides.* The labeled mercuric reductase preparation that was used for sequencing retained 10% of the original mercuric reductase activity (23 $min^{-1}$) and contained 0.84 $^{14}C$ label per FAD. It was hoped that substoichiometric labeling would maximize the specificity of the iodoacetamide alkylation of $EH_2$. Radiolabeled mercuric reductase was prepared and digested with trypsin; the resulting peptides were separated by HPLC as described under Experimental Procedures. Two major radiolabeled peptide peaks were obtained, accounting for 70 and 20% of the applied radioactivity (Figure 3). Essentially no change in the HPLC profile was seen between a 60-min and a 22-h tryptic digestion. The *mer*A gene sequence indicates that 49 peptides should be produced from a tryptic digestion (Brown et al., 1983), and at least 40 of these can be clearly distinguished in the HPLC chromatograph.

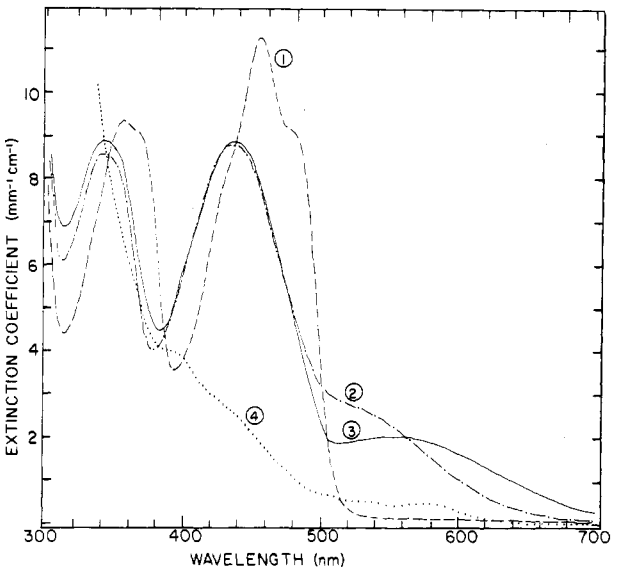



FIGURE 2: Spectrum of monoalkylated derivative of mercuric reductase. An anaerobic solution of 30.8 nmol of mercuric reductase in 1 mL of 80 mM sodium phosphate, pH 7.4–0.2 mM EDTA was titrated to the $EH_2$ level with $Na_2S_2O_4$ as described under Experimental Procedures. EHR was generated by adding 100 nmol of iodoacetamide, and the spectrum was recorded immediately after the addition. The spectrum of $EH_4$ was generated in a separate experiment by the addition of an excess of $Na_2S_2O_4$ (Fox & Walsh, 1982). (1) E; (2) $EH_2$; (3) EHR; (4) $EH_4$.

Table III: Amino Acid Sequence of the Active Site of Labeled Mercuric Reductase[a]

| cycle | major PTH-amino acid identified | run A nmol | run A cpm[b] | run B nmol | run B cpm |
|---|---|---|---|---|---|
| 1 | Gly[c] | 0.97 | | 1.15 | |
| 2 | Thr[d] | | | | |
| 3 | Ile | 1.85 | | 0.58 | |
| 4 | Gly | 0.40 | | 0.47 | |
| 5 | Gly | 0.50 | | 0.38 | |
| 6 | Thr | | 104 | | 90 |
| 7 | SCMC[e] | | 7580 | | 1700 |
| 8 | Val | 0.75 | 770 | 0.40 | 660 |
| 9 | Asx | 0.54 | 330 | 0.36 | 40 |
| 10 | Val | 0.50 | 70 | 0.45 | 30 |
| 11 | Gly[f] | 0.27 | | | |
| 12 | SCMC | | 430 | | 90 |
| 13 | Val | 0.40 | | 0.27 | |
| 14 | Pro (?)[g] | | | | |

[a] Two samples, A and B, originating from separate tryptic digestions, were sequenced as described under Experimental Procedures, with repetitive yields of 86 and 92%, respectively. [b] The radioactivity in each residue is given as the value over background levels. Approximately 50% of each sample was counted. The ratio of label at fraction 7 to fraction 12 was calculated after adjusting for the repetitive yield. [c] Gly was identified by the presence of PTH-Gly and PTC-Gly. The quantitation is based on PTH-Gly alone and is therefore an underestimate. [d] Thr was identified by the absorbance of the dehydro-PTH-Thr at 313 nm, eluting slightly later than PTH-SCMC, as well as peaks absorbing at 254 nm. It was not possible to reliably quantitate the Thr due to the multiplicity of products. [e] Cys was identified by the absorbance of the PTH derivative of the alkylated cysteine at 313 nm and by the presence of radioactivity. [f] No value is listed for run B as the peak was too small to be picked up by the integrator. [g] The PTH-Pro peaks were too small to be picked up by the integrator.

*Sequence of the Active Site Peptide.* The sequence of the active site peptide is shown in Table III. The gene sequence, which became available to us at the time of protein sequencing, indicated that the presumed active site tryptic peptide should contain 16 amino acids. The peptide sequence that was de-

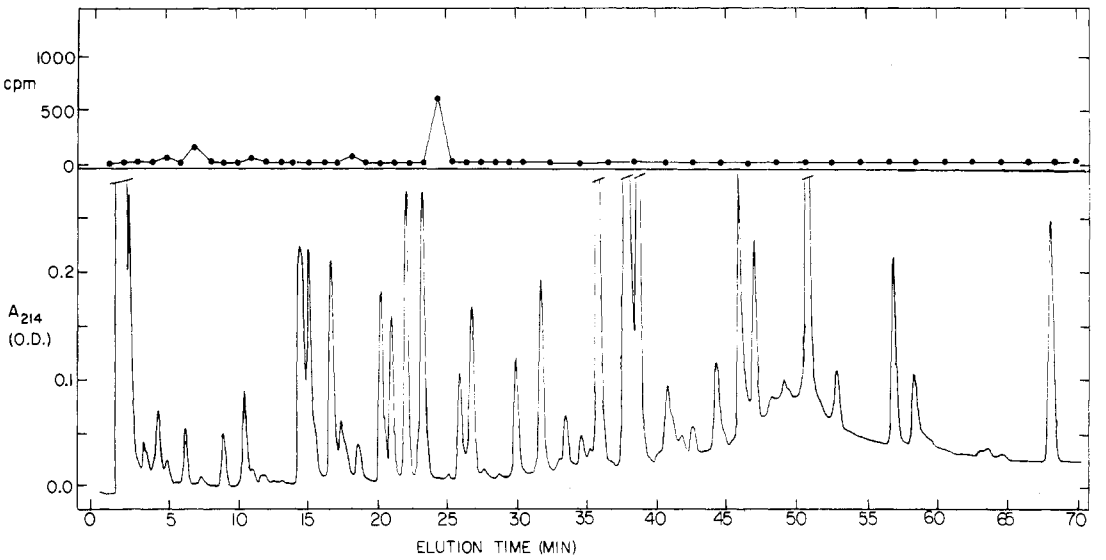



FIGURE 3: HPLC separation of tryptic peptides of mercuric reductase. Approximately 14 nmol of labeled mercuric reductase in 300 μL of 100 mM $NH_4HCO_3$, pH 8.1, was digested with TPCK-trypsin (final concentration 10 μg/mL) at room temperature for 24 h. A 200-μL fraction of this reaction mixture was separated on reverse-phase HPLC as described under Experimental Procedures. Two-milliliter fractions were collected, and 0.05-mL aliquots were removed for scintillation counting. The top graph, showing the radioactivity, has not been adjusted to account for the delay of 1–2 mL (0.5–1.0 min) between the UV detector and the fraction collector.

termined is consistent with the amino acid composition (data not shown) and the above predictions from the DNA sequence (Brown et al., 1983). The specific activity of the peptide (the peptide concentration was measured by amino acid composition) was 80% of the specific activity of the iodo[$^{14}$C]acetamide used for the alkylation, a figure that agrees well with the 84% efficiency of inactivation. Both alkylated cysteine residues contained some radioactive label, as indicated in Table III. Adjusting for the repetitive yield of sequencing, it is seen that the alkylation of the cysteine closer to the $NH_2$ terminus of the peptide, termed Cys-T7, was overwhelmingly preferred by a factor of 18 ± 1.

*Composition of the Minor Labeled Peptide.* The minor alkylated peptide, containing 20% of the total radioactivity, was subjected to amino acid analysis. It contained Ala (1.1), SCMC (1.1), Glu (0.8), Gly (1.0), Leu (0.7), and Ser (0.8). This compares favorably with the composition of the COOH-terminal tryptic peptide predicted by the gene sequence Glu-Leu-Ser-Cys-Cys-Ala-Gly (Brown et al., 1983). It was not determined whether both cysteines were labeled. The isolation of this labeled peptide demonstrates that no posttranslational COOH-terminal processing of mercuric reductase has occurred and that the COOH terminus is accessible to electrophiles when the enzyme has been reduced by two electrons.

## Discussion

Mercuric reductase is the most recently described member of the small class of flavoproteins that contain a catalytically active cystine residue in the active site. Previous work in this laboratory has suggested that it is mechanistically similar to the other enzymes of this type, the nicotinamide disulfide oxidoreductases (Fox & Walsh, 1982). In this paper, data are reported that demonstrate the structural similarities of the active site peptides of these enzymes.

The extent of active site sequence homology between mercuric reductase, glutathione reductase, and lipoamide dehydrogenase is remarkable (Figure 4). A comparison of mercuric reductase and either glutathione reductase (human erythrocytes or yeast) or lipoamide dehydrogenase (*Escherichia coli*) reveals absolute identity in 12 or 11 out of the 16 residues in the active site, respectively. In the last 15 amino acids of the peptide, only one residue differs from that seen

| | |
|---|---|
| Mercuric Reductase | Gly Thr Ile Gly Gly Thr Cys Val Asn Val Gly Cys Val Pro Ser Lys |
| Glutathione Reductase | His Lys Leu Gly Gly Thr Cys Val Asn Val Gly Cys Val Pro Lys Lys |
| Lipoamide Dehydrogenase | Asn Thr Leu Gly Gly Val Cys Leu Asn Val Gly Cys Ile Pro Ser Lys |

FIGURE 4: Active site sequences of mercuric reductase and the nicotinamide disulfide oxidoreductases. The identification of the COOH-terminal Ser-Lys of the mercuric reductase sequence is based on the amino acid composition and the DNA sequence (Brown et al., 1983); the identification of Asn rather than Asx is also based on the DNA sequence. The sequences shown for glutathione reductase and lipoamide dehydrogenase were determined from enzyme isolated from human erythrocytes (Untucht-Grau et al., 1981) and *E. coli* (Brown & Perham, 1972; Burleigh & Williams, 1972), respectively. In lipoamide dehydrogenase from pig heart, Val-6 is replaced by Thr (Matthews et al., 1974).

in either glutathione reductase or lipoamide dehydrogenase; in mercuric reductase, an Ile replaces a Leu. In all three enzymes, the two redox-active cysteines are separated by four amino acids. The sequence of this peptide, particularly the presence of three invariant glycines and one proline, provides a strong indication of homology in the secondary and tertiary structure as well.

The similarity in the response of mercuric reductase, glutathione reductase, and lipoamide dehydrogenase to alkylation is also striking. The active site Cys-T7 residue of mercuric reductase is approximately 18-fold more reactive with iodoacetamide than is Cys-T12. Alkylation of pig heart lipoamide dehydrogenase and yeast glutathione reductase results in the same cysteine being preferentially labeled by a factor of 13 and 8, respectively (Thorpe & Williams, 1976a; Arscott et al., 1981).

The active site sequence homology between mercuric reductase and the nicotinamide disulfide oxidoreductases is the most compelling evidence to date that these enzymes may be related. To this must be added the similar reactivity with iodoacetamide and the previously reported physical and spectroscopic similarities (Fox & Walsh, 1982). In addition, the enzymes catalyze the oxidation of nicotinamides with an identical stereospecificity, indicating a common relative orientation of binding. Together, these data argue strongly for a similar active site organization. A great deal of information is available on the structure of the active sites of glutathione reductase and lipoamide dehydrogenase. Most importantly, the crystal structure of glutathione reductase, solved at 2-Å

resolution (Thieme et al., 1981; Schulz et al., 1982), indicates that the nicotinamide binding site and the redox-active disulfide lie on opposite faces of the flavin moiety. Electrons pass sequentially from the reduced nicotinamide to the flavin and from the flavin to the disulfide, presumably via a 4a-flavin adduct (Thorpe & Williams, 1976b). Consistent with the iodoacetamide labeling results, the crystal structure also shows that it is the cysteine closer to the COOH terminus, Cys-T12, that is involved in electron transfer with the flavin (Schulz et al., 1978; Williams et al., 1978); Cys-T7 functions in substrate binding and is therefore more accessible to solvent and iodoacetamide. In the $EH_2$ form of the enzyme, Cys-T12 forms a charge-transfer complex with the oxidized flavin, giving the enzyme the long-wavelength absorbance characteristic for this flavoprotein class.

Generalizing this active site structure to mercuric reductase, it becomes possible to interpret the catalytic and spectral properties of the monoalkylated enzyme. Alkylation of Cys-T7 prevents binding and reduction of mercuric ions. Modification at this site with iodoacetamide, however, would not be expected to directly affect the nicotinamide binding site. This is confirmed by the finding that transhydrogenase activity in mercuric reductase is not inhibited by iodoacetamide labeling. Transhydrogenase activity actually increases upon alkylation, in analogy to the behavior of lipoamide dehydrogenase, suggesting the induction of a conformational change that indirectly affects this second site.

Alkylation with iodoacetamide induces a variety of spectral changes in these three similar enzymes. In glutathione reductase, the spectrum of EHR is virtually indistinguishable from that of $EH_2$ (Arscott et al., 1981), supporting the model in which the charge-transfer complex is formed between Cys-T12 and the oxidized flavin. In the spectrum of monoalkylated lipoamide dehydrogenase, however, no charge-transfer band is seen, and the spectrum closely resembles that of E, oxidized enzyme (Thorpe & Williams, 1976a). It was thought that the alkylation of this enzyme altered the orientation of Cys-T12, destroying the charge-transfer complex (Arscott et al., 1981). Active site specific monoalkylation of mercuric reductase resulted in the formation of a new spectral species with an absorbance maximum centered around 580 nm. This spectrum of EHR closely resembles that seen under several different circumstances with lipoamide dehydrogenase. The first is when a complex is formed between $EH_2$ and $NAD^+$ (Matthews et al., 1976); the other is the complex between EHR and the nicotinamide analogue $AAD^+$ (Thorpe & Williams, 1981). It has been postulated that in lipoamide dehydrogenase this spectrum is due to a modified charge-transfer complex between Cys-T12 and oxidized flavin (Thorpe & Williams, 1981). The binding of $AAD^+$ to EHR or of $NAD^+$ to $EH_2$ may change the orientation of Cys-T12. This conformational change can apparently be induced in other ways as well; a similar spectrum is observed with *E. coli* lipoamide dehydrogenase $EH_2$ in the presence of 0.2 M guanidinium chloride (Wilkinson & Williams, 1979). With mercuric reductase, the observed spectrum is induced not by binding of nicotinamides or guanidinium chloride but by thiol alkylation.

This spectral change in the EHR form of mercuric reductase seems to be complete at a time when only 50% of the subunits have been monoalkylated. This suggests communication between the subunits of this dimeric enzyme. Support for intersubunit communication comes from an examination of the structure of glutathione reductase. It is seen that the two active sites in the dimer are formed by structural components of both subunits (Schulz et al., 1978). Furthermore, in studies examining the binding of $NAD^+$ to lipoamide dehydrogenase EHR (Thorpe & Williams, 1981), it was found that only one $NAD^+$ bound per dimer with the concomitant induction of C-4a adduct formation between flavin and thiol. It was hypothesized that the $NAD^+$ bound to one subunit and induced a conformational change, lowering the affinity of the second site in the dimer for the nicotinamide. With mercuric reductase, it appears that thiol alkylation may be inducing a similar conformational change. Alkylation of one thiol per dimer is apparently sufficient to perturb the spectrum of the charge-transfer complex and to stimulate the transhydrogenase activity at the other active site.

The active site cysteine residues in mercuric reductase, Cys-T7 and Cys-T12, are found at positions 136 and 141, respectively, of the amino acid sequence (Brown et al., 1983). In glutathione reductase from human erythrocytes and in lipoamide dehydrogenase from pig heart, however, the cysteines are found at positions 58 and 63 (Untucht-Grau et al., 1981) and 45 and 50 (C. H. Williams, personal communication), respectively. These data suggest the presence of an additional $NH_2$-terminal segment in mercuric reductase. The fact that limited digestion of mercuric reductase with chymotrypsin and trypsin produces fragments of similar molecular weights demonstrates that one region of the enzyme is uniquely susceptible to proteolysis. The location of the chymotryptic clip has been determined to be following Trp-85, indicating the presence of a discrete $NH_2$-terminal domain. The chymotryptic clip places the active site cysteine residues of mercuric reductase at positions 51 and 56, now in register with the active sites of glutathione reductase and lipoamide dehydrogenase. The removal of 85 amino acids from mercuric reductase also reduces its molecular weight from 59 000 (Brown et al., 1983) to 51 000, comparable to the molecular weights reported for lipoamide dehydrogenase and glutathione reductase (51 000–53 000) (Williams, 1976). No catalytic role has been found for this domain in vitro.

The COOH terminus of mercuric reductase also differs from lipoamide dehydrogenase and glutathione reductase. The isolated fragment contains two cysteine residues, and the ease of labeling of two-electron-reduced enzyme by iodoacetamide suggests that it is readily accessible to electrophiles. The COOH-terminal region of the structurally similar enzyme glutathione reductase is involved in glutathione binding (Schulz et al., 1982). It is therefore tempting to postulate a role for these cysteines in substrate binding, either at the active site or prior to exchange of the $Hg^{2+}$ onto the active site thiols.

This paper has reported on the extensive sequence homology and functional similarities in the active sites of mercuric reductase and the nicotinamide disulfide oxidoreductases. The findings reported here should provide insight into the evolution and adaptation of enzyme structure. Coupled with the availability of the Tn501 *mer*A gene sequence, these studies set the stage for a wider comparison of mercuric reductase with the primary structure of lipoamide dehydrogenase and the primary and tertiary structures of glutathione reductase. Furthermore, the extent of the homology between these enzymes suggests that X-ray crystallography of mercuric reductase may aid in the identification of the structural features that provide the unique catalytic ability of mercuric reductase to reduce $Hg^{2+}$.

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**Registry No.** NADPH, 53-57-6; mercuric reductase, 67880-93-7; lipoamide dehydrogenase, 9001-18-7; glutathione reductase, 9001-48-3; L-cystine, 56-89-3; iodoacetamide, 144-48-9; L-cysteine, 52-90-4.

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