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C-Terminal Cysteines of Tn*501* Mercuric Ion Reductase†

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ABSTRACT: Mercuric ion reductase (MerA) catalyzes the reduction of Hg(II) to Hg(0) as the last step in the bacterial mercury detoxification pathway. A member of the flavin disulfide oxidoreductase family, MerA contains an FAD prosthetic group and redox-active disulfide in its active site. However, the presence of these two moieties is not sufficient for catalytic Hg(II) reduction, as other enzyme family members are potently inhibited by mercurials. We have previously identified a second pair of active site cysteines (Cys_{558} Cys_{559} in the Tn*501* enzyme) unique to MerA, that are essential for high levels of mercuric ion reductase activity [Moore, M. J., & Walsh, C. T. (1989) *Biochemistry 28*, 1183; Miller, S. M., et al. (1989) *Biochemistry 28*, 1194]. In this paper, we have examined the individual roles of Cys_{558} and Cys_{559} by site-directed mutagenesis of each to alanine. Phenotypic analysis indicates that both *merA* mutations result in a total disruption of the Hg(II) detoxification pathway in vivo, while characterization of the purified mutant enzymes in vitro shows each to have differential effects on catalytic function. Compared to wild-type enzyme, the C558A mutant shows a 20-fold reduction in k_{cat} and a 10-fold increase in K_m, for an overall decrease in catalytic efficiency of 200-fold in k_{cat}/K_m. In contrast, mutation of Cys_{559} to alanine results in less than a 2-fold reduction in k_{cat} and an increase in K_m of only 4-5-fold for an overall decrease in catalytic efficiency of only ca. 10-fold in vitro. From these results, it appears that Cys_{558} plays a more important role in forming the reducible complex with Hg(II), while both Cys_{558} and Cys_{559} seem to be involved in efficient scavenging (i.e., tight binding) of Hg(II).

The extraordinarily high affinity of mercuric ions for thiols, and thus proteins, renders them exceedingly toxic to living systems. Since several hundred million tons of mercurials are dispersed throughout the biosphere, it is not surprising that microorganisms have evolved a variety of means for their detoxification [for review see Foster (1987), Summers (1986), and Summers and Silver (1978)]. By far the most common resistance mechanism is a strategy of reductive biotransformation. In this system, Hg(II) is scavenged from the immediate microenvironment and transported into the cytoplasm by the sequential action of two gene products, MerP and

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MerT. Inside the cell, the enzyme, mercuric ion reductase (MerA),[1] catalyzes the reduction of Hg(II) to Hg(0) at the expense of NADPH. Once reduced, the lipophilicity and high vapor pressure of elemental mercury promote its unassisted diffusion out of the cell and away from the immediate vicinity. Bacteria that are resistant to organomercurials encode a second enzyme, organomercurial lyase (MerB), which catalyzes the protonolytic fragmentation of C–Hg bonds to yield Hg(II) and the unsubstituted hydrocarbon [for review see Moore et al. (1990), Summers (1986), and Brown (1985)].

Mercuric reductase is one of the very rare enzymes that catalyzes a reaction utilizing a metal substrate. It is an α_2-homodimer and belongs to the flavin disulfide oxidoreductase family that includes glutathione reductase, lipoamide dehydrogenase, and trypanothione reductase. However, while all members of the family have homologous active sites containing an FAD cofactor and a redox-active disulfide, only mercuric reductase can reduce, and thereby detoxify, mercuric ions. In fact, other members of the family are potently inhibited by mercurials (Massey & Williams, 1965; Cassola & Massey, 1966).

We have previously shown that, in addition to the redox-active disulfide, a unique feature of the mercuric reductase active site is the presence of a second pair of essential cysteines (Moore & Walsh, 1989; Miller et al., 1989). This pair, Cys_{558} Cys_{559} in the Tn*501* enzyme, is located near the extreme C-terminus of the protein and is donated to the active site by the subunit opposite to that which contributes the redox-active disulfide (Distefano et al., 1990). Site-directed mutagenesis of both Cys_{558} and Cys_{559} to alanines (to form the CCAA mutant enzyme) resulted in a 1000-fold decrease in the rate of Hg(II) reduction (Moore & Walsh, 1989). Efficient Hg(II) reduction by the wild-type enzyme requires that Cys_{558} and Cys_{559} be present as thiols; the enzyme as isolated contains a disulfide between Cys_{558} and Cys_{559} which must be reduced to activate mercuric reductase activity (Miller et al., 1989).

While the results of the above studies clearly indicated the importance of the C-terminal cysteines in Hg(II) reduction, it was not previously possible to define their individual contributions to the active site. In this paper, we report the construction of two new mutant enzymes, CCAC (Ala_{558}) and CCCA (Ala_{559}), in which each of the C-terminal cysteines has been changed singly to an alanine. Phenotypic analysis has shown that both mutations have very similar and profound effects upon the function of the entire mercury detoxification pathway in vivo. In vitro characterization of the purified enzymes, however, indicates differential roles for the two cysteines: removal of Cys_{559} served primarily to reduce the affinity of the enzyme for Hg(II) (higher K_m), while removal of Cys_{558} adversely affected both k_{cat} and K_m. The relationship of these results to the recently solved X-ray crystal structure of mercuric reductase from *Bacillus* sp. RC607 (Schiering et al., 1991a,b) and the in vivo coupling between Hg(II) transport and Hg(II) reduction are discussed.

[1] Abbreviations: amp, ampicillin; C10A-C13A, Ala_{10} Ala_{13} mercuric reductase; CCAA, Cys_{135} Cys_{140} Ala_{558} Ala_{559} mercuric reductase; CCAC, Cys_{135} Cys_{140} Ala_{558} Cys_{559} mercuric reductase; CCCA, Cys_{135} Cys_{140} Cys_{558} Ala_{559} mercuric reductase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; EH_2, mercuric reductase containing oxidized FAD and reduced active site thiols; EH_4, mercuric reductase containing $FADH_2$ and reduced active site thiols; EOP, efficiency of plating; E_{ox}, mercuric reductase containing oxidized FAD and active site disulfide; FAD, flavin adenine dinucleotide; IPTG, isopropyl β-D-thiogalactopyranoside; kDa, kilodalton; LB, Luria-Bertani media; MerA, mercuric reductase; MIC, minimum inhibitory concentration; $NADP^+$, oxidized β-nicotinamide adenine dinucleotide phosphate; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; thio$NADP^+$, thionicotinamide adenine dinucleotide phosphate; TNB^-, 5-thio-2-nitrobenzoate dianion.

Experimental Procedures

Materials

Restriction enzymes and other DNA modification enzymes were obtained from New England Biolabs, Bethesda Research Laboratories, or Boehringer Mannheim. Avian reverse transcriptase was purchased from Pharmacia, Klenow was from New England Biolabs or Amersham, and Sequenase was obtained from United States Biochemicals. Radiolabeled nucleotides were purchased from Amersham or New England Nuclear. Oligonucleotides were synthesized on a Pharmacia Gene Assembler DNA synthesizer using β-amidite reagents from Pharmacia. *Escherichia coli* strains W3110 *lacI*^{q} (Schultz et al., 1985), HB101*, JM101, and RZ1032, M13 derivative M13mm1, and plasmids pUB3451, pMMa558a559, pMMOa558a559, pSE181, and pMMOa10a13 have been described previously (Moore & Walsh, 1989). All other reagents and chemicals were of the highest grade commercially available and were used without further purification.

Methods

Routine DNA manipulations were performed as described by Maniatis et al. (1982). Transformations were performed according to the simple transformation protocol of Hanahan (1985). Double-stranded plasmid DNA was sequenced with avian reverse transcriptase (Seidman, 1986), while single-stranded DNA was sequenced with Klenow (Amersham, 1983) or Sequenase (United States Biochemicals, 1988). UV–visible absorbance spectra were recorded with a Hewlett-Packard 8452A diode array spectrophotometer. Fluorescence spectra were recorded with a scanning ratio spectrofluorometer built by Gordon Ford and Dr. David Ballou at the University of Michigan. Rapid-reaction studies were carried out by measuring absorbances with a stopped-flow spectrophotometer with scanning capabilities (2-cm optical path) as previously described (Beaty & Ballou, 1981). Rapid kinetic data were analyzed using a Marquardt-Levenberg routine in a program called *A* developed at the University of Michigan by Rong Chang, Chung-Jen Chiu, and Dr. David Ballou. Anaerobic titrations were carried out in anaerobic cuvettes similar to those previously described (Williams et al., 1979). Solutions were made anaerobic by repeated evacuation followed by flushing with Argon passed over an R & D oxygen trap (R & D Separations) and an Oxisorb (Messer Griesheim) indicator column. Protein thiols were titrated either as previously described (Miller et al., 1989) (but aerobically) or, more conveniently, by dilution of 10–100 μL of enzyme stock to 1 mL with 8 M guanidine, pH 7.8, containing 5 mM EDTA, followed by addition of 50 μL of ethanolic 10 mM DTNB. Thiol concentrations were calculated using $\epsilon_{412} = 13.6\ mM^{-1}\ cm^{-1}$ for TNB^- (Beutler et al., 1963).

Mutagenesis. The Ala_{558} Cys_{559} (CCAC) and Cys_{558} Ala_{559} (CCCA) mutations were generated in the M13mp19 derivative, M13mm1 (Moore & Walsh, 1989), using single-stranded template DNA that had been prepared in the *dut*$^-$ *ung*$^-$ host strain RZ1032 (Kunkel, 1985; Kunkel et al., 1987). The mutagenesis primers, 5′-GGCGCAGGCGGAAAGCTG-CTT-3′ and 5′-CCCGGCGGCGCAGGAAAGCTGCTT-3′, respectively, each contained two mismatched base pairs (underlined). Mutagenesis reactions (using primer:template ratios of 10:1) were as described by Kunkel (1985) except that the uracil glycosylase treatment was omitted. After transformation

of the reaction mixtures into *E. coli* strain JM101, phage isolated from the resulting plaques were screened either by dot-blot hybridization (Zoller & Smith, 1983) followed by Sanger sequencing (CCAC) or by direct sequencing of the first round phage isolates (CCCA). By this method, the mutant derivatives, M13mma558 and M13mma559, were obtained in 1.3% and 11.1% yields, respectively.

Plasmid Constructions. (A) pMMa558 and pMMa559. The CCAC and CCCA mutant *merA* genes were initially reconstructed within the context of an otherwise intact *mer* operon. This was accomplished by replacing the 775 bp *Sph*I/*Bam*HI fragment of pUB3451 with the same fragment from either M13mma558 or M13mma559, to yield plasmids pMMa558 and pMMa559, respectively. [A more detailed account of these manipulations has been presented elsewhere for the construction of pMMa558a559 (containing the CCAA mutant *merA*; Moore & Walsh, 1989)]. After pMMa558 and pMMa559 had been confirmed by restriction mapping and sequencing in the area of the desired mutations, the same 775-bp *Sph*I/*Bam*HI fragment of each that had undergone mutagenesis was subcloned back into M13mp19 and completely resequenced to eliminate the possibility that any undesired, secondary mutations had resulted from the mutagenesis pocedure.

(B) pMMOa558 and pMMOa559. Overexpression vectors for the CCAC and CCCA mutant MerA proteins were constructed by inserting the *merA*-containing 1861-bp *Aha*II fragment of either pMMa558 or pMMa559 behind the *tac* promotor of plasmid pSE181. This generated plasmids pMMOa558 and pMMOa559, respectively. These manipulations exactly paralleled those used to construct the CCAA MerA overproducer, pMMOa558a559, from pMMa558a559 and pSE181 (Moore & Walsh, 1989).

Minimum Inhibitory Concentration Assays. Cultures of *E. coli* HB101* harboring the desired plasmids were grown to log phase in LB medium supplemented with 50 μg/mL ampicillin (amp^+ LB). Small aliquots (<10 μL) of each culture were struck onto sectors of amp^+ LB agar plates containing from 0 to 50 μM $HgCl_2$ in 1 μM increments. After incubation at ambient temperature for two days, plates were scored for colony size and distribution (Foster et al., 1979; Ni'Bhriain et al., 1983). HB101* (pUB3451) cells were also assayed for growth on plates containing 100 and 150 μM $HgCl_2$.

Efficiency of Plating Assays. E. coli HB101 or HB101* cells containing the appropriate plasmids were grown overnight in amp^+ LB medium. These cultures were serially diluted 10^1–10^8-fold into fresh LB medium, and 100 μL from each dilution was spread in triplicate onto LB agar plates containing ampicillin (100 μg/mL) and various concentrations of $HgCl_2$. Plates were incubated for 3–5 days at ambient temperature before scoring for colony formation. The efficiency of plating at each $HgCl_2$ concentration was taken to be the ratio of the cells surviving at that $HgCl_2$ concentration to the total number of viable cells in the original culture (i.e., the number of colonies on the plates containing antibiotic only) (Ni'Bhriain et al., 1983; Ross et al., 1989).

Protein Purification. Wild-type and mutant enzymes were overexpressed in *E. coli* W3110 $lacI^q$ cells harboring the appropriate overexpression plasmid. Protein induction and purification was carried out as previously described (Miller et al., 1989) except that prior to OrangeA chromatography, the cell lysate was supplemented with ca. 50 μM FAD. Before use, all enzyme samples were treated with 2 M KBr to remove tightly bound $NADP^+$ (Miller et al., 1989).

Results

Phenotypic Analysis. The in vivo effects of the CCAC and CCCA mutations were assessed by examining the $HgCl_2$ resistance phenotypes of cells expressing each mutant *merA* allele within the context of an otherwise wild-type *mer* operon. Resistance levels were first determined using the minimum inhibitory concentration (MIC) assay. This assay, which measures the minimum $HgCl_2$ concentration required to inhibit cell growth, was performed by streaking out log phase cultures of HB101* cells harboring pUB3451, pMMa558, pMMa559, pMMa558a559, or pSE181 onto agar plates containing ampicillin and various concentrations of mercuric ion (Foster et al., 1979; Ni'Bhriain et al., 1983). After incubation at ambient temperature for 2 days, the lowest $HgCl_2$ concentration at which normal colony size and distribution became inhibited was taken to be the MIC.

Under the above conditions, the MIC for cells carrying pUB3451 (wild-type *mer* operon) was between 100 and 150 μM $HgCl_2$. This level of tolerance to mercuric ions, exhibited by cells expressing both a functional Hg(II)-transport system and an intact mercuric reductase, is termed wild type or fully *Hg(II)-resistant*. In contrast, the MIC for cells harboring pSE181 (an amp^R mer^- control) was 31 μM $HgCl_2$. These cells express neither the Hg(II)-transport proteins nor mercuric reductase and are said to be *Hg(II)-sensitive*. A third phenotypic class, *Hg(II)-supersensitive*, is typified by cells carrying pMMa558a559 (CCAA *merA*). These bacteria retain the Hg(II)-specific transport activity but lack the ability to detoxify it; the CCAA mutant mercuric reductase is at least 1000-fold less efficient at Hg(II) reduction than is the wild-type enzyme (Moore & Walsh, 1989). They essentially commit suicide by concentrating Hg(II) in their cytoplasms and can be adversely affected by $HgCl_2$ concentrations as low as 2.5 μM (Moore & Walsh, 1989). Under the conditions employed here, pMMa558a559 cells grew normally at 4 μM $HgCl_2$ but were substantially inhibited by 5 μM $HgCl_2$ (Figure 1).

The MIC's of pMMa558 (CCAC *merA*) and pMMa559 (CCCA *merA*) cells are also illustrated in Figure 1. Like bacteria carrying pMMa558a559, these cells flourished at 4 μM $HgCl_2$, but their growth was severely retarded by 5 μM $HgCl_2$. In addition, for all three plasmids even the small, scattered colonies observable on the 5 μM $HgCl_2$ plates were totally absent on plates containing 19–22 μM $HgCl_2$ or above. Therefore, we can conclude that both pMMa558 and pMMa559 encode the same phenotype as pMMa558a559; i.e., their presence causes cells to become *fully supersensitive* to mercuric ions.

Another measure of phenotype is the efficiency of plating (EOP) assay, which quantifies the actual percentage of viable cells at Hg(II) concentrations above the MIC. This test is extremely sensitive and has been used to distinguish small variations within the main phenotypic classes (Rose et al., 1989). EOP data for *E. coli* harboring pSE181, pMMa558, pMMa559, or pMMa558a559 are summarized in Table I. As expected from the MIC results, growth of pSE181 cells was essentially unaffected (EOP ratios near 1.0) by $HgCl_2$ concentrations up to 25 μM. However, at 30 and 50 μM $HgCl_2$ the survivability of pSE181 cells was substantially decreased by greater than 300- and 3400-fold, respectively. In contrast, the growth of bacteria harboring pMMa558, pMMa559, or pMMa558a559 was strongly inhibited at all five $HgCl_2$ concentrations tested, with EOP ratios varying from $<1.2 \times 10^{-4}$ at 15 μM $HgCl_2$ to $<1.2 \times 10^{-6}$ at 50 μM. At every $HgCl_2$ concentration tested the survival ratios of all three supersen-

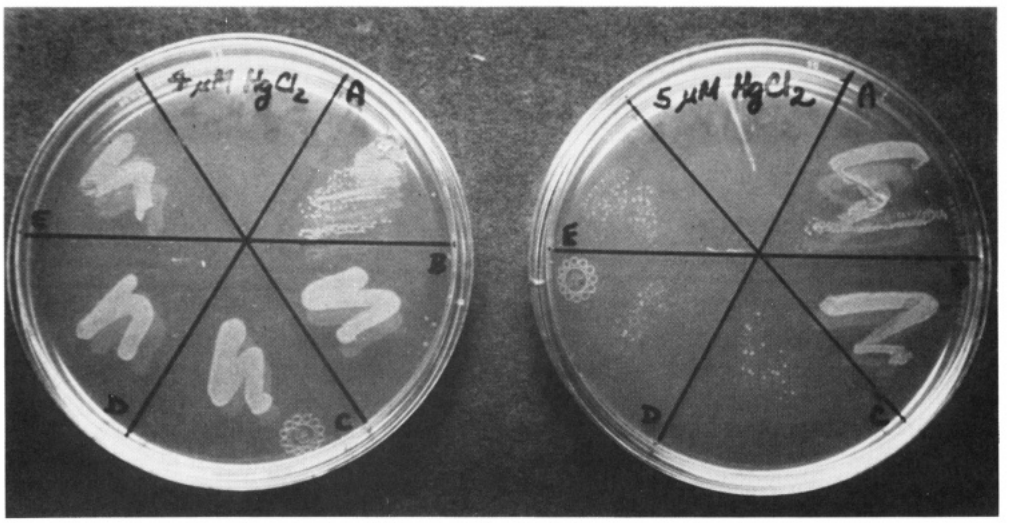

FIGURE 1: Minimum inhibitory concentration (MIC) assay for *E. coli* expressing wild-type and C-terminal mutant mercuric reductases from plasmids containing otherwise intact *mer* operons. Sectors are labeled clockwise: (A) pUB3451 (wild type), (B) pSE181 (*mer*$^-$ control), (C) pMMa558a559 (CCAA MerA), (D) pMMa558 (CCAC MerA), (E) pMMa559 (CCCA MerA). The photograph shows 4 (left) and 5 μM (right) $HgCl_2$ plates after 2 days of growth at ambient temperature.

Table I: Efficiencies of Plating for *E. coli* Harboring pSE181, pMMa558, pMMa559, or pMMa558a559

μM $HgCl_2$	pSE181	pMMa558	pMMa559	pMMa558 a559
15	0.99 ± 0.41	$<1.4 \times 10^{-4}$ [b]	$<1.3 \times 10^{-4}$ [b]	$<1.2 \times 10^{-4}$ [b]
20	0.97 ± 0.32	$(4.1 \pm 1.5) \times 10^{-5}$	$(0.8 \pm 0.1) \times 10^{-5}$	$(3.9 \pm 1.3) \times 10^{-5}$
25	0.93 ± 0.30	$(3.2 \pm 1.6) \times 10^{-5}$	$(1.8 \pm 0.9) \times 10^{-5}$	$(2.5 \pm 0.8) \times 10^{-5}$
30	<0.6, <0.003[a]	$(3.4 \pm 1.4) \times 10^{-5}$	$(0.7 \pm 1.0) \times 10^{-5}$	$(0.8 \pm 0.1) \times 10^{-5}$
50	$<2.9 \times 10^{-6}$ [b]	$<1.4 \times 10^{-6}$ [b]	$<1.3 \times 10^{-6}$ [b]	$<1.2 \times 10^{-6}$ [b]

[a] Only upper limits are given because 10^4, 10^5, and 10^6 dilutions of the original overnight culture gave no colonies, while lesser dilutions yielded >2000 colonies/100 μL. Such phenomenology was commonly observed when cells were plated at high density and is presumed to result from nonspecific adsorption of Hg(II) to the cells, effectively lowering the $HgCl_2$ concentration on the surface of the agar plate. This effect is necessarily greater the higher the cell density, and at some density it may result in lowering the Hg(II) concentration to nonlethal levels. In the case of pSE181 at 30 μM $HgCl_2$, the <0.6 value was calculated from the 10^4 dilution data where many colonies were observed, while the <0.003 value was calculated from the 10^5 dilution data given that 1 colony per plate (which is the smallest observable signal) was not obtained, thus defining the upper limit of the plating efficiency. For the reasons given above, the authors believe that the <0.003 value is a better estimation of the true plating efficiency than is the <0.6 value. [b] Only upper limits are given because no colonies were observed at a 10^2 dilution of the original culture, the lowest dilution tested. 10^0 and 10^1 dilutions were not plated for the reasons noted in footnote *a*.

sitive strains were quite similar. Thus, in accordance with the MIC data, the EOP results also indicate that both pMMa558 and pMMa559 encode fully supersensitive phenotypes in vivo.

Protein Overproduction and Purification. To facilitate protein purification, the CCAC and CCCA mutant *merA* genes were cloned behind the tac promotor (Amann et al., 1983) of plasmid pSE181 as previously described for the CCAA mutant (Moore & Walsh, 1989). The resulting plasmids, pMMOa558 and pMMOa559, respectively, were used to transform *E. coli* W3110 *lacI*^{q} cells, where MerA expression was induced with IPTG, a gratuitous inducer of tac. Both plasmids directed significant overexpression of their respective enzymes, yielding MerA as 18.8% and 9.1% total cell protein for pMMOa558 and pMMOa559, respectively (as estimated by scanning laser densitometry of an SDS–polyacrylamide gel; data not shown). These expression levels represent 23- and 11-fold enhancements, respectively, over wild-type MerA expression from the *mer* operon context of pJOE114 (Schultz et al., 1985) and are somewhat higher than that produced by a similar tac-based wild-type overproducer (5% soluble cell protein; Schultz et al., 1985).

Wild-type and mutant MerA proteins were isolated in >100-mg quantities using the purification scheme described by Miller et al. (1989). The only modification of this procedure in the present work has been the addition of excess FAD to the crude cell lysate prior to OrangeA affinity chromatography. We have observed that this serves to increase enzyme yields, apparently because protein overexpression depletes endogenous FAD resulting in significant apoprotein production. SDS–PAGE analysis of the purified proteins revealed that the preparations were >95% pure, containing one major (59 kDa) and two minor bands (ca. 50 kDa; data not shown). The two minor bands are known proteolytic products of full-length enzyme in which the removal of an 85 amino acid, N-terminal domain has no adverse effects on enzyme activity (Fox & Walsh, 1982, 1983).

Thiol Titrations. Immediately prior to any other form of characterization, each enzyme sample was examined for its thiol content. These titrations were performed for two reasons. First, since the mutations under consideration in this study are at the extreme C-terminus of the enzyme (positions 558 and 559 out of 561 total amino acids) and the N-terminus of mercuric reductase is known to be particularly prone to proteolytic removal [see above and Fox and Walsh (1982, 1983)], it was desirable to ensure that the C-terminal cysteine(s) in the sample under study had not been lost to proteolysis. Second, the C-terminal cysteine pair of wild-type enzyme is known to be quite sensitive to oxidation (see below), and this oxidation affects both enzyme fluorescence and mercuric reductase activity levels (Miller et al., 1989; Sandstrom & Lindskog, 1987). Thus, it is of primary importance to know the thiol content of any mercuric reductase sample at all times.

Thiol titration of the CCAC and CCCA mutant enzymes immediately after purification and treatment with KBr (see Methods) yielded respective thiol titers of 5.3 and 4.9 per enzyme monomer. Since both mutant genes encode seven total cysteines, and the redox-active disulfide between Cys_{135} and

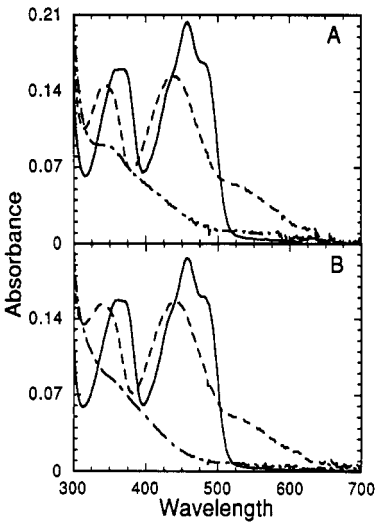

FIGURE 2: UV–visible absorption spectra of the CCAC and CCCA mutant mercuric reductases. The anaerobic enzymes in 50 mM potassium phosphate buffer, pH 7.3, were titrated with sodium dithionite in the same buffer at 4 °C. (Panel A) 17.8 μM CCAC mutant; (panel B) 17.7 μM CCCA mutant; (—) E_{ox}, (---) EH_2, (-·-) EH_4.

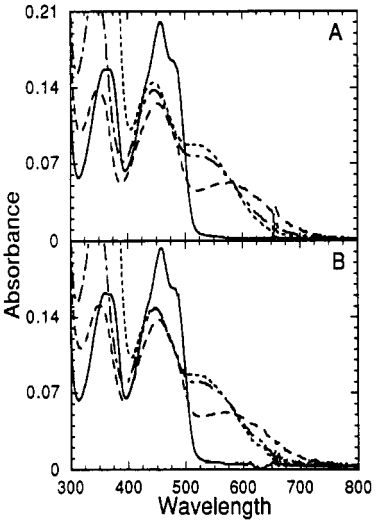

FIGURE 3: Pyridine nucleotide complexes of the CCAC and CCCA mutant mercuric reductases. The anaerobic enzymes in 50 mM potassium phosphate buffer, pH 7.3, were titrated with a solution of 2.1 mM NADPH in 2 mM unneutralized Tris at 4 °C. (Panel A) 17.7 μM CCAC; (panel B) 17.9 μM CCCA; (—) 0 equiv, (--) 1.2 equiv, (-·-) 2.4 equiv, (---) 13.2 equiv of NADPH/FAD.

Cys_{140} is fully formed in both (as evidenced from their visible absorbance spectra below), these data indicate that both the CCAC and CCCA proteins retain their full complement of cysteinyl thiols upon purification. It should be noted that similarly treated wild-type enzyme contains only 3.8 titratable thiols [see Table II in Miller et al. (1989)] out of a total of eight cysteines per monomer. This shortfall is due to the formation of a second, auxiliary disulfide in wild-type enzyme, between Cys_{558} and Cys_{559} (Miller et al., 1989). However, since both the CCAC and CCCA mutants lack one of these C-terminal cysteinyl residues, neither is capable of forming this auxiliary disulfide.

Spectroscopic Properties. Visible absorbance spectra of the CCAC and CCCA mutant proteins are presented in Figure 2. Like wild-type mercuric reductase and other members of this oxidoreductase family, in the absence of nicotinamides both mutants can exist in three spectrally distinct redox states: fully oxidized (E_{ox}), two-electron reduced (EH_2), and four-electron reduced (EH_4). E_{ox}, in which both the flavin and redox-active disulfide (between Cys_{135} and Cys_{140}) are oxidized, appears distinctly yellow and exhibits a well-resolved spectrum having a characteristic λ_{max} at 456 nm (ϵ = 11.3 mM^{-1} cm^{-1}). In the EH_2 state the disulfide is reduced while the flavin remain oxidized. This causes the enzyme to appear reddish-brown due to long-wavelength charge-transfer absorbance ($\lambda_{max} \approx$ 535 nm; ϵ = 2.8 mM^{-1} cm^{-1}) from interaction of the Cys_{140} thiolate with the flavin. The EH_4 state is characterized by a bleached spectrum signaling flavin, as well as disulfide, reduction.

In all three redox states, the spectra of the CCAC and CCCA mutants are remarkably similar to those of wild-type enzyme, having identical absorbance maxima and extinction coefficients. In fact, we have previously observed that even a double mutation at the C-terminus, CCAA, causes no perturbation in these absorbance spectra (Moore & Walsh, 1989). Thus, compared to Cys_{135} and Cys_{140} [see Schultz et al. (1985) and Distefano et al. (1989)] the C-terminal cysteines must not be in intimate contact with the flavin.

In contrast to the case of absorbance, the C-terminal cysteines are known to have a dramatic effect on enzyme fluorescence. In wild-type mercuric reductase, reduction of the auxiliary disulfide between Cys_{558} and Cys_{559} causes a marked decrease in the fluorescence intensity of enzyme-bound flavin. Thus, at 2 °C and pH 7.3, "nonactivated E_{ox}" (auxiliary disulfide) fluoresces 6.3 times as intensely as free FAD, whereas "activated E_{ox}" (auxiliary dithiol) is only 2.6 times as fluorescent. Removal of both cysteines gives the CCAA mutant the same fluorescence intensity as nonactivated wild type E_{ox}: 6.3 times free FAD [see Table I in Miller et al. (1989)]. Interestingly, the single Cys to Ala mutants exhibit differential fluorescence intensities. The CCAC mutant is 5.8 times more fluorescent than free FAD at pH 7.3, similar to CCAA and nonactivated wild-type enzyme. However, the CCCA mutant is 4.5 times more fluorescent than FAD at pH 7.3, suggesting some influence by Cys_{558} on the fluorescence intensity but no influence by Cys_{559}. As with the absorbance properties, neither mutation affects the excitation (456 nm) or emission (520 nm) maxima nor the general shape of the fluorescence spectrum (data not shown).

Nicotinamide Binding Properties. Results of anaerobic titrations of the CCAC and CCCA mutant enzymes with NADPH are shown in Figure 3. As seen with wild-type enzyme (Miller et al., 1989) and the CCAA mutant (Moore & Walsh, 1989), addition of ca. 1 equiv of NADPH/FAD to E_{ox} results in reduction of the redox-active disulfide and formation of an EH_2–$NADP^+$ complex with long-wavelength absorbance ($\lambda_{max} \approx$ 580 nm). Further addition of NADPH leads to displacement of $NADP^+$ by NADPH yielding an EH_2–NADPH complex where the long-wavelength band shifts back to ca. 535 nm but with approximately twice the extinction of free EH_2. Note that most of the enzyme is converted to the EH_2–NADPH complex by addition of the second equivalent of NADPH/FAD, indicating overall tighter binding of NADPH than $NADP^+$ to EH_2; however, complete displacement of $NADP^+$ requires excess NADPH. This is slightly more pronounced in the case of the CCAC enzyme, where both the long-wavelength band and the 450-nm region peak increase upon addition of the excess NADPH. This has also been seen with wild-type enzyme (S. M. Miller, unpublished results) in contrast to the earlier reports by Sahlman et al. (1984) of no difference between the complex formation upon reaction of E_{ox} with 2 or 10 equiv of NADPH. Overall, these mutants behave the same as wild-type enzyme with only subtle, if any, differences in their affinities for pyridine nucleotides.

Table II: Oxidase and Hg(II)-Reductase Activities of Wild-Type and Mutant Mercuric Reductases As Measured by NADPH Consumption Assays

		Hg(II)-reductase	
enzyme	oxidase[a]	aerobic[b]	anaerobic[c]
wild type	2.0 min^{-1}	$K_{m\,Hg(II)}$ = 5.0 μM k_{cat} = 810 min^{-1}	$K_{m\,Hg(II)}$ = 5.7 μM k_{cat} = 446 min^{-1}
CCCA	3.1 min^{-1}	$K_{m\,Hg(II)}$ = 25 μM k_{cat} = 458 min^{-1}	$K_{m\,Hg(II)}$ = 21 μM k_{cat} = 291 min^{-1}
CCAC	1.0 min^{-1}	$K_{m\,Hg(II)}$ = 45 μM k_{cat} = 30 min^{-1}	$K_{m\,Hg(II)}$ = 52 μM k_{cat} = 23 min^{-1}

[a] Oxidase activity was measured at 37 °C in air-saturated 80 mM sodium phosphate buffer, pH 7.4, containing 1 mM 2-mercaptoethanol and 200 μM NADPH. NADPH consumption was monitored at 340 nm ($\Delta\epsilon$ = 6.2 mM^{-1} cm^{-1}). [b] Aerobic assays were performed at 37 °C in 80 mM sodium phosphate buffer, pH 7.4, containing 1 mM 2-mercaptoethanol, 200 μM NADPH, and various concentrations of $HgCl_2$. Reactions were initiated with $HgCl_2$ after the Hg(II)-independent NADPH oxidase rate was established. Initial velocities were taken as the Hg(II)-stimulated NADPH oxidase rate minus the background rate. [c] Anaerobic assays were performed at 25 °C using a stopped-flow spectrophotometer with a 2-cm observation cell. Anaerobic solutions of the dilute enzymes in 50 mM potassium phosphate buffer, pH 7.3, were mixed with equal volumes of anaerobic substrate solutions in the same buffer to give final concentrations of 1 mM 2-mercaptoethanol, 25 μM NADPH, varied $HgCl_2$, and 25 nM enzyme monomer for the CCCA and C10A-C13A (a wild-type equivalent) mutant enzymes or 200 nM enzyme monomer for the CCAC mutant.

The kinetics of reduction of the E_{ox} form of the CCAC and CCCA mutant enzymes by NADPH were briefly examined using a stopped-flow spectrophotometer. When mixed with 8 equiv of NADPH/FAD at pH 7.3 and 4 °C, both enzymes exhibited absorbance changes in the dead time as well as two further phases at the several wavelengths examined (e.g., 430, 480, 525, and 600 nm). Analysis of the two observable phases gives apparent rate constants of 27 s^{-1} and 6.1 s^{-1} for the CCCA enzyme and 51 s^{-1} and 5.8 s^{-1} for the CCAC enzyme. These results are in good agreement with those reported by Sahlman et al. (1984) for wild-type enzyme, where appearance of the EH_2–$NADP^+$ complex occurs with an apparent rate constant of 43 s^{-1} followed by exchange of NADPH for $NADP^+$ to form the EH_2–NADPH complex with an apparent rate constant of 7.8 s^{-1}. Thus, neither the thermodynamics nor the kinetics of the interactions of these mutant enzymes with pyridine nucleotides appear to be significantly altered relative to those interactions of wild-type enzyme.

Catalytic Properties. Wild-type and mutant mercuric reductases catalyze several reactions in addition to the reduction of $Hg(SR)_2$. These include transhydrogenation between NADPH and thio-$NADP^+$, reduction of the aryl disulfide, DTNB, and reduction of dioxygen in aerobic solutions (Schultz et al., 1985; Distefano et al., 1989; Moore et al., 1989). In the present study, we have examined the abilities of the wild type and mutants to reduce both dioxygen and mercuric ions, and these data are summarized in Table II.

O_2 Reduction. In the absence of any other electron acceptor, mercuric reductase will transfer electrons from NADPH to dioxygen to form H_2O_2. In air-saturated buffer at 37 °C, wild-type enzyme catalyzes this reaction with a turnover number of 2.0 min^{-1}/enzyme monomer (Schultz et al., 1985). All mutant enzymes characterized to date (including the redox-active disulfide mutants) retain this activity, having turnover numbers from 0.2 to 56 min^{-1} which can be correlated with flavin redox potential (Distefano et al., 1989). The CCAA mutant, with an EH_2/EH_4 midpoint potential close to that of wild-type enzyme, also has an oxidase turnover number of 2.0 min^{-1} (Moore & Walsh, 1989). The CCAC and CCCA mutants are quite similar with turnover numbers of 1.0 and 3.1 min^{-1}, respectively.

Hg(II) Reduction. As an initial estimate of activity of the mutant enzymes relative to wild type, we determined k_{cat} and $K_{m\,Hg(II)}$ values at 37 °C under aerobic conditions in the presence of 1 mM 2-mercaptoethanol. (There was no dependence of the rate on NADPH concentration from 5 to 200 μM for any of the enzymes.) The results, summarized in Table II, suggest that the CCCA mutant still has a fairly high turnover number (at least 50% that of wild type), but the K_m for Hg(II) is elevated 5-fold, meaning the enzyme will be much slower when the concentration of mercuric ions is low. In the case of the CCAC enzyme, the turnover number is only 4% that of wild-type enzyme while the K_m for Hg(II) is elevated 9-fold; i.e., this mutant appears to be more severely disabled.

While the aerobic assays provided some insight into the effects of the mutagenesis on Hg(II)-reductase activity, there remained a significant uncertainty in the turnover numbers, particularly for the CCAC enzyme with its low turnover, since it has been found that some mutants exhibit a Hg(II)-stimulated O_2-reductase (oxidase) activity [for discussion see Distefano et al. (1989)]. Therefore, we have examined the ability of the C10A-C13A mutant enzyme (a wild-type equivalent) and CCAC and CCCA mutants to reduce Hg(II) under strictly anaerobic conditions, using a stopped-flow spectrophotometer cell as an anaerobic chamber. As summarized in Table II, in the presence of 1 mM 2-mercaptoethanol at 25 °C, wild-type mercuric reductase exhibited a K_m for mercuric ions of 5.7 μM, with k_{cat} = 446 min^{-1}; CCCA enzyme exhibited a K_m for Hg(II) of 21 μM, with k_{cat} = 291 min^{-1} (65%); and CCAC enzyme exhibited a K_m for Hg(II) of 52 μM, with k_{cat} = 23 min^{-1} (5%). All of the K_m values are in good agreement with those obtained aerobically, while all of the turnover numbers are somewhat lower. The apparent lower activity may be due in part to the absence of oxygen (particularly in the case of the CCAC enzyme), but may also result from partial inactivation of the enzymes which occurs upon making these rather dilute solutions anaerobic.

The above results clearly indicate that removal of the Cys_{558} thiol (CCAC mutant) severely reduces the catalytic efficiency of the enzyme toward Hg(II) reduction. However, the CCCA mutant does not seem to be so grossly affected in vitro even though it cannot efficiently detoxify the Hg(II) in vivo. In an attempt to address this discrepancy, we measured the activity of the CCCA enzyme using cysteine as a better model for the (probably glutathione-like) in vivo ligand for Hg(II) instead of 2-mercaptoethanol. Sandstrom and Linskog (1987) report values for wild-type enzyme of $K_{m\,Hg(II)}$ = 3.2 μM and k_{cat} = 810 min^{-1} in the presence of 1 mM cysteine at 25 °C (aerobic). Under identical conditions (except anaerobically), we obtained values for the CCCA enzyme of $K_{m\,Hg(II)}$ = 350 μM and k_{cat} = 450 min^{-1}.

As an additional test of the role of the individual cysteines in Hg(II) binding, we have examined the effect of varying the free thiol concentration on the apparent activity. We have previously shown that wild-type enzyme is inhibited at both low and high thiol concentrations, where the low concentration effect is presumably due to complexation of more than one Hg(II) per active site, and the high concentration effect (primarily a K_m effect) results from significant reversal of Hg(II) binding steps (Miller et al., 1986). Figure 4 summarizes the results of experiments where substrate concentrations of oxidized enzyme were rapidly mixed with ca. 8 equiv of NADPH/FAD, 6 equiv of $HgCl_2$/FAD, and various concentrations of 2-mercaptoethanol using a stopped-flow spectrophotometer. Two aspects of the results should be em-

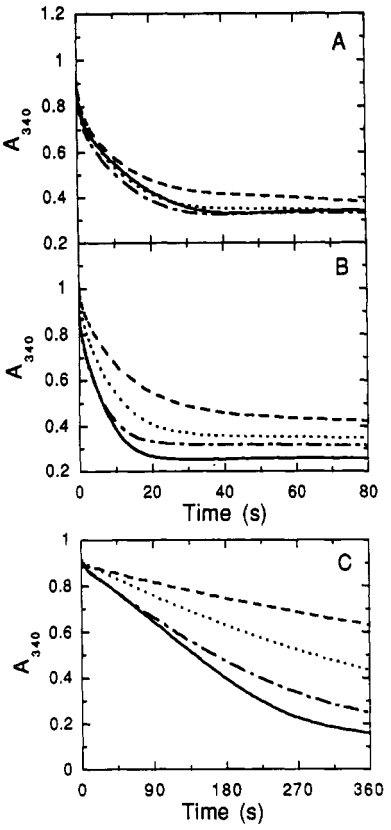

FIGURE 4: Effect of thiol concentration on turnover. Anaerobic solutions of oxidized enzymes in 50 mM potassium phosphate buffer, pH 7.3, were mixed with equal volumes of solutions containing NADPH, $HgCl_2$, and varied 2-mercaptoethanol in the same buffer, using a stopped-flow spectrophotometer at 4 °C. Final concentrations: 75 μM NADPH; 55 μM $HgCl_2$; (—) 0.2 mM, (–·–) 0.5 mM, (···), 1.0 mM, (– –) 2.5 mM 2-mercaptoethanol. (Panel A) 9.1 μM wild type; (panel B) 9.3 μM CCCA mutant; (panel C) 10.6 μM CCAC mutant.

phasized. First, the time scale for the six turnovers by the wild-type and CCCA enzymes is the same (80 s), indicating that the CCCA mutant can indeed reduce Hg(II) at a rate similar to that of wild-type enzyme. However, the time scale for the CCAC enzyme is much longer (360 s), indicating a much lower rate of turnover. Second, since the absorbance scales are the same for all panels, we can see by inspection that while all three enzymes show a lower apparent rate of turnover as the free thiol concentration is increased, both the CCCA and the CCAC enzymes show a much greater sensitivity to the free thiol concentration than does the wild-type enzyme. Thus, both mutant enzymes have a decreased ability relative to wild-type enzyme to compete with free thiol for mercuric ion. It should also be noted that at the lowest thiol concentration examined (0.2 mM) the wild-type enzyme becomes slightly inhibited as discussed above, while the CCCA enzyme actually exhibits a higher apparent turnover number than the wild-type enzyme, 78 min^{-1} vs 25 min^{-1} (4 °C), respectively.

Discussion

In its wild-type context, mercuric reductase (MerA) functions as part of an integrated Hg(II)-detoxification pathway, which includes specific regulatory and transport activities in addition to the reductase. In this system, toxic Hg(II) ions are actually pumped into the cell where reducing equivalents are readily available to the reductase in the form of NADPH. Obviously, for this pathway to be effective at detoxification, rates of transport and reduction must be tightly coupled, with rates of reduction preferably exceeding those of transport. Additionally, the reductase must rapidly sequester the incoming Hg(II) to prevent complexation with other cellular proteins. It is expected then that mutations compromising either the reductive ability or the binding capacity of the protein may lead to an "uncoupling" of the pathway, where rates of transport exceed the effective rate of turnover by the reductase. In this view, it is both relevant and necessary to determine the effects of any mutation in MerA on the operation of the intact detoxification system in vivo, as well as on the isolated enzyme in vitro.

We have previously demonstrated that mutagenesis of both C-terminal cysteines to alanines (CCAA mutant) results in a supersensitive phenotype toward Hg(II) when the mutated gene is incorporated into the otherwise intact Tn*501 mer* operon and expressed in vivo. Furthermore, the isolated CCAA mutant enzyme exhibits only 0.1% of the wild-type activity, indicating that the in vivo effect is indeed due to an overall lower rate of Hg(II) reduction by the mutant (Moore & Walsh, 1989). However, the results presented here for the CCAC and CCCA mutations suggest a more complex picture for the roles of the individual cysteines. With both mutations, indistinguishable supersensitive phenotypes are exhibited upon expression of the mutant reductase within the otherwise intact operon. Clearly, the absence of either Cys_{558} or Cys_{559} renders the reductase incapable of efficiently detoxifying Hg(II) within cells. However, from the phenotypic data alone, we could not differentiate between effects due to a loss of efficient Hg(II) reduction and those due to a loss of effective competition by the reductase with other cellular proteins for binding Hg(II). This distinction comes from the in vitro characterization.

Isolation of the CCAC and CCCA mutant proteins proceeded normally as with wild type and most of the mutants generated thus far (Schultz et al., 1985; Distefano et al., 1989; Moore & Walsh, 1989), suggesting no significant effect of the mutations on the pyridine nucleotide binding properties of the enzyme. Indeed, NADPH titrations show formation of the same complexes seen with wild-type enzyme with nearly identical stoichiometries. Additionally, the formation rates of these complexes are similar to those observed with wild-type enzyme, indicating that neither mutagenesis has significantly altered the ability of the enzyme to catalyze the transfer of electrons from pyridine nucleotide through the flavin to the redox-active disulfide on the other side. We have previously shown that the CCAA mutant exhibits similar spectral and nicotinamide binding properties (Moore & Walsh, 1989; Miller et al., 1989). Thus, the C-terminal cysteine to alanine mutations seem to affect only Hg(II) binding and its subsequent reduction by the enzyme.

The X-ray crystal structure of mercuric reductase from marine *Bacillus* sp. RC607 has recently been solved to 3.0-Å resolution (Schiering et al., 1991a,b). While the Tn*501* and *Bacillus* enzymes share only 41% overall amino acid identity (Wang et al., 1989), the sequences surrounding the catalytic core residues have been remarkably conserved. In the *Bacillus* enzyme, the redox-active disulfide is formed between Cys_{207} and Cys_{212}, while Cys_{628} and Cys_{629} comprise the C-terminal pair. The X-ray structure reveals that in the NADPH-reduced enzyme, the C-terminal cysteines do indeed reside in the active site, with the Cys_{628} thiol disposed 5.2 Å away from the Cys_{207} thiol and the Cys_{629} thiol pointing outward from the active site. (The Cys_{212} thiolate interacts with the C4a position of the flavin ring in a charge-transfer complex.) Although no Hg(II) crystal derivatives have yet been obtained, the structure of Cd(II) bound in the active site of pyridine nucleotide reduced

enzyme has been determined. In this complex the Cd(II) is bound in a distorted tetrahedron with the thiolates from Cys_{207} and Cys_{628}, as well as the hydroxyl groups of two tyrosines, Tyr_{264} and Tyr_{605}, as proposed ligands (Schiering et al., 1991b). These tyrosines are conserved in the Tn*501* enzyme, being Tyr_{194} and Tyr_{545}, respectively.

The X-ray crystal structure results are consistent with the mutant enzyme studies reported here. Since the C-terminal cysteines are not in intimate contact with the flavin, as are the redox-active cysteines, their mutagenesis would not be expected to greatly affect the spectral properties of the isoalloxazine ring, which is our observation. The fact that Cys_{558} can affect the fluorescence intensity of the flavin while Cys_{559} cannot seems reasonable given their respective dispositions within the active site (Cys_{558} = Cys_{628} folded in and Cys_{559} = Cys_{629} folded out). Additionally, our observations concerning the catalytic properties of the cysteine to alanine mutations largely agree with the structural data. Removal of the Cys_{559} thiol caused less than a 2-fold reduction in k_{cat} for Hg(II) reduction, while removal of the Cys_{558} thiol caused a 20–25-fold reduction in k_{cat}. Again, these results are entirely consistent with the structural data since Cys_{558} (Cys_{628} in *Bacillus*) is clearly positioned to participate with the redox-active thiols to form some type of complex with Hg(II) near the flavin (from which electrons flow), whereas Cys_{559} (Cys_{629} in *Bacillus*) is turned away in the reduced crystal. The effects on the K_m values for Hg(II) are also fairly consistent with the structural data since removal of the Cys_{558} thiol has a more detrimental effect than removal of the Cys_{559} thiol (increases of 10-fold vs 4–5-fold, respectively). However, the effects on K_m and k_{cat}, as well as the increased sensitivity of the CCCA mutant to free thiol concentrations in turnover, would not necessarily be predicted from the structure and indicate that there is likely to be a fair amount of flexibility of the C-terminus in solution that allows the Cys_{559} thiol to approach the other residues of the active site more closely. This hypothesis is also supported by the fact that the C-terminal cysteines form a disulfide upon storage at 4 °C (Miller et al., 1989), which was indeed present in the crystal structure of the oxidized enzyme (Schiering et al., 1991b).

The dramatic effects of the mutations on the kinetic properties of the enzyme coupled with the new structural information, particularly of the Cd(II) complex, make comparison of the metal ion binding properties of the mutant and wild-type enzymes a high priority. Preliminary binding studies have been carried out by observing Hg(II)-induced changes in the visible charge-transfer absorption band due to interaction between Cys_{140} and the flavin in EH_2–$NADP^+$ enzyme complexes [such complexes can bind but not reduce Hg(II)]. These experiments have already revealed clear differences between the wild-type and mutant enzymes. For example, while 1 equiv of Hg(II) per active site completely quenches the charge-transfer band in the CCAA and CCAC mutant enzymes, it only partially quenches the band in wild-type enzyme, and it causes the band to shift to longer wavelength in the CCCA mutant (S. M. Miller, unpublished results). These results indicate that both Cys_{558} and Cys_{559} surely participate in Hg(II) binding in the wild-type enzyme, but the structural aspects are not yet clear. EXAFS studies are currently being pursued to obtain structural information on a variety of Hg(II) and Cd(II) complexes of both wild-type and mutant enzymes.

Coming back to the analysis of the in vivo effects in terms of the in vitro catalytic parameters, the 20–25-fold reduction in k_{cat} for the CCAC mutant seems a sufficient reason to expect uncoupling of the reductase from transport. However, the less than 2-fold decrease in k_{cat} for the CCCA enzyme cannot entirely explain why a fully supersensitive phenotype is observed with this mutant. If we consider the overall catalytic efficiency in terms of k_{cat}/K_m, we find that the CCAC enzyme is ca. 180–240-fold less efficient and the CCCA enzyme is ca. 6–9-fold less efficient than wild-type enzyme in the in vitro assays with 2-mercaptoethanol. This criterion further clarifies the ineptness of the CCAC mutant in vivo but still leaves some uncertainty as to why the CCCA mutant is so supersensitive in vivo. Considering the toxicity of Hg(II), one might expect a little more built in flexibility in the coupling of transport and reductase efficiencies in vivo. A more telling result, however, is the increase in $K_{m\,Hg(II)}$ for the CCCA enzyme from 21 μM in the presence of 2-mercaptoethanol to 350 μM in the presence of cysteine with no change in k_{cat} relative to wild-type enzyme. Under these conditions, the overall catalytic efficiency (k_{cat}/K_m) of the CCCA enzyme is ca. 60-fold lower than that of wild-type enzyme, a result much more consistent with the fully supersensitive phenotype. Clearly, an interesting experiment to shed more light on the coupling of activities in this pathway would be to incorporate "slower" mutants of the transport protein with the CCCA reductase mutant to look for return of viability.

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Registry No. Cys, 52-90-4; $HgCl_2$, 7487-94-7; MerA, 67880-93-7.

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A Low Molecular Weight Platelet Inhibitor of Factor XIa: Purification, Characterization, and Possible Role in Blood Coagulation†

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ABSTRACT: A low molecular weight platelet inhibitor of factor XIa (PIXI) has been purified 250-fold from releasates of washed and stimulated human platelets. Molecular weight estimates of 8400 and 8500 were determined by gel filtration and SDS–polyacrylamide gel electrophoresis, respectively, although a second band of M_r 5000 was present upon electrophoresis. The inhibitor does not appear to be one of the platelet-specific, heparin-binding proteins, since it neither bound to nor was affected by heparin. An amount of PIXI which inhibited by 50% factor XIa cleavage of the chromogenic substrate S2366 (Pyr-Glu-Pro-Arg-pNA·$2H_2O$) only slightly inhibited (5–9%) factor XIIa, plasma kallikrein, plasmin, and activated protein C and did not inhibit factor Xa, thrombin, tPA, or trypsin, suggesting specificity for factor XIa. Kinetic analyses of the effect of PIXI on factor XIa activity demonstrated mixed-type, noncompetitive inhibition of S2366 cleavage and of factor IX activation with K_i's of 7×10^{-8} and 3.8×10^{-9} M, respectively. Immunoblot analysis showed that PIXI is not the inhibitory domain of protease nexin II, a potent inhibitor of factor XIa also secreted from platelets. Amino acid analysis showed that PIXI has no cysteine residues and, therefore, is not a Kunitz-type inhibitor. PIXI can prevent stable complex formation between α_1-protease inhibitor and factor XIa light chain as demonstrated by SDS–polyacrylamide gel electrophoresis. The inhibition by PIXI of factor XIa-catalyzed activation of factor IX and its capacity to prevent factor XIa inactivation by α_1-protease inhibitor, combined with the specificity of PIXI for factor XIa among serine proteases found in blood, suggest a role for PIXI in the regulation of intrinsic coagulation.

Control of intrinsic coagulation may be exerted most effectively by regulation of factor XIa activity, since factor XIa appears to be the first enzyme in this coagulation pathway required for normal hemostasis (Rosenthal et al., 1953; Ragni et al., 1985). Factor XIa, a serine protease produced in blood from zymogen factor XI, is a dimeric molecule of two identical, disulfide-linked, polypeptide chains (M_r 80000), each of which has a M_r 50000 heavy chain linked to a M_r 30000 light chain by disulfide bridges (Bouma & Griffin, 1977; Kurachi & Davie, 1977). The light chain contains the catalytic site (Kurachi & Davie, 1977; van der Graaf et al., 1983), whereas the heavy chain has sites for the binding surface (Mannhalter & Schiffman, 1980), a cofactor (high molecular weight kininogen) (van der Graaf et al., 1983; Sinha et al., 1985; Baglia et al., 1989, 1990), and its substrate factor IX (Sinha et al.,

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