Structures of the Reduced and Mercury-Bound Forms of MerP, the Periplasmic Protein from the Bacterial Mercury Detoxification System†,$‡

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ABSTRACT: Bacteria carrying plasmids with the mer operon, which encodes the proteins responsible for the bacterial mercury detoxification system, have the ability to transport Hg(II) across the cell membrane into the cytoplasm where it is reduced to Hg(0). This is significant because metallic mercury is relatively nontoxic and volatile and can be passively eliminated. The structures of the reduced and mercury-bound forms of merP, the periplasmic protein, which binds Hg(II) and transfers it to the membrane transport protein merT, have been determined in aqueous solution by multidimensional NMR spectroscopy. The 72-residue merP protein has a $\beta\alpha\beta\beta$ fold with the two $\alpha$ helices overlaying a four-strand antiparallel $\beta$ sheet. Structural differences between the reduced and mercury-bound forms of merP are localized to the metal binding loop containing the consensus sequence GMDXXC. The structure of the mercury-bound form of merP shows that Hg(II) is bicoordinate with the Cys side chain ligands, and this is confirmed by the chemical shift frequency of the $^{199}$Hg resonance.

There are enormous amounts of heavy metals in the environment. Organometallic compounds resulting from various types of industrial and military waste are of particular concern. These metals and their compounds are nearly universally toxic to biological organisms including humans because of their nonselective chemistry; for example, since Hg(II) reacts with essentially all exposed sulfhydryl groups on proteins, it interferes with a wide range of biological functions (Walsh et al., 1988). Therefore, it is surprising to find that some bacteria thrive in the presence of high concentrations of heavy metal toxins. This is possible only because these bacteria possess efficient mechanisms for the detoxification of heavy metals (Summers et al., 1986). A plausible explanation for the presence of these mechanisms is that since the earth’s prebiotic environment was undoubtedly heavily polluted with heavy metals from geochemical processes, the most primitive organisms had to evolve ways for dealing with heavy metals so that Cys, His, and other amino acids with side chains capable of binding to metals could be utilized in their proteins. Regardless of the initial sources, genes associated with bacterial resistance to a wide variety of toxic metals have been described (Silver, 1992; Silver & Waldeshau, 1995).

The most thoroughly investigated bacterial mercury detoxification system is remarkable. It functions by transporting toxic Hg(II) into the cell where it is converted to relatively nontoxic metallic Hg(0) which is volatile and can be passively eliminated (Brown, 1985; Foster, 1987; Summers, 1986). The sequences of the proteins responsible for mercury detoxification are encoded in the mer operon on a plasmid that typically also has operons that confer antibiotic resistance (Foster, 1987). The mer operon consists of several structural genes whose expression is regulated by the merR repressor protein (O’Halloran et al., 1993). The merP (periplasm) protein binds mercury in the periplasm and transfers it to the merT (transport) protein responsible for transporting mercury through the membrane into the cytoplasm (Brown, 1985; Lund & Brown, 1987). Other components of the system include the enzymes mercuric reductase, which reduces Hg(II) to Hg(0) in the cytoplasm (Schiering et al., 1991), and organomercury lyase (Foster, 1987), which removes organic ligands from Hg(II).

In addition to their direct biological toxicity through damage to proteins, heavy metals are involved in several human diseases where transport functions are perturbed. For example, both Menkes and Wilson diseases result from improper copper metabolism, and the genes responsible for these diseases have been shown to correspond to P-type ATPases containing multiple repeats of a metal binding domain with sequences highly homologous to that of merP (Lutsenko & Kaplan, 1995). Indeed, mutations in the merP-like domains of these proteins are associated with human diseases (Chelly et al., 1993; Mercer et al., 1993). Further, a possible connection has been found between the mercury in dental amalgam fillings and antibiotic resistance in oral and intestinal bacteria because those bacteria with plasmids containing both mer and antibiotic resistance operons are

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‡ Coordinates have been deposited in the Brookhaven Protein Data Bank (filenames 1afi and 1ajj).
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1 Abbreviations: CBCACOH, C$^3$ carbon to C$^6$ to carbonyl carbon to amide proton correlation; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FHSQC, fast heteronuclear single-quantum coherence; HMQC, heteronuclear multiple-quantum coherence; HNHA, amide proton to nitrogen to C$^3$H proton correlation; HNCA, amide proton to nitrogen to C$^3$H proton coherence; HMQC, heteronuclear multiple-quantum coherence; HNHA, amide proton to nitrogen to C$^3$H proton coherence; HMQC, heteronuclear multiple-quantum coherence; HNCA, amide proton to nitrogen to C$^3$H proton coherence; HNCO, heteronuclear single-quantum coherence; IPTG, isopropyl thiogalactoside; MBP, maltose binding protein; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; PCR, polymerase chain reaction; rmsd, root mean square deviation; TOSY, total correlation spectroscopy; TPPI, time-proportional phase incrementation; SA, simulated annealing.

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selected by the release of mercury from the dental fillings (Lorscheider et al., 1995). Interest in these proteins also results from their potential use in bioremediation, including their expression in plants for removal of heavy metals in soils (Rugh et al., 1996).

We are using NMR spectroscopy to determine the structures of the proteins responsible for the initial recognition, binding, and transport of mercury through the cell membrane in the bacterial mercury detoxification system. MerP and merT, in particular, are attractive candidates for structural studies because they are small, with 72 residues (97 as expressed) for merP and 116 residues (122 as expressed) for merT. MerP is soluble in aqueous solution and gives well-resolved two- and three-dimensional NMR spectra, enabling its structure to be determined by multidimensional solution NMR spectroscopy as described in this paper. MerT, which is an intrinsic membrane protein, requires a combination of solution NMR and solid-state NMR methods for its structure determination in micelle and bilayer environments (Opella, 1994). The studies of merP and merT, in combination with the ongoing structural studies on the merR repressor (Ansari et al., 1992) and mercuric reductase (Schiering et al., 1991) and organomercury lyase enzymes (Ralston & O’Halloran, 1990; Helmann et al., 1990), should provide considerable insight into the chemistry and structural biology of the bacterial detoxification system. The secondary structure and overall fold of oxidized merP in solution determined by $^1$H NMR spectroscopy has been previously reported (Eriksson & Sahlman, 1993). This paper describes the three-dimensional structures of the reduced and mercury-bound forms of merP in solution determined by heteronuclear multidimensional NMR spectroscopy.

**MATERIALS AND METHODS**

**Materials.** Oligonucleotide primers were synthesized by the University of Pennsylvania Cancer Center Nucleic Acid Facility. The plasmid pHK1 containing the merP gene was provided by Nancy Hamlett, assisted by Matt Harris and Dave Karlton (Swarthmore College). Competent DH5$\alpha$ Escherichia coli cells were purchased from GibcoBRL (Gaithersburg, MD), and cells of strain BL21/BL21(DE3) were purchased from Novagen (Madison, WI). The plasmid vector pMAL-c2, protease factor Xa, amylose affinity resin, and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). Restriction enzymes EcoRI and HindIII were purchased from GibcoBRL (Gaithersburg, MD). A Sequenase version 2.0 DNA sequencing kit was purchased from Amersham (Buckinghamshire, UK). The column was washed with 3 volumes of 20 mM sodium phosphate containing 1 g/L (15 NH$_4$)$_2$SO$_4$ and 2 g/L unlabeled glucose) or by using 13C/15N-labeled algam media. A 1 mL culture in rich LB media was used to inoculate 25 mL of minimal media which was grown overnight. This was then used to inoculate 1 L of minimal media. The bacteria were grown to an OD$_{550}$ of about 0.5 before induction with IPTG (final concentration 0.5 mM). Incubation was continued for 2–3 h before being harvested by centrifugation. All cultures were maintained at 37 °C and contained 100 µg/mL ampicillin. Cells were immediately frozen and kept at −80 °C until further use.

**Construction of Expression Vector pSSS.** The merP gene was amplified by PCR from pHK1 by using forward and reverse primers. The plasmid pHK1 is a construct containing the merP gene from the transposon Tn2 (on plasmid R100) which originated from a Shigella flexneri strain of bacteria found in Japan in 1959 (Misra et al., 1984). The sequence for the forward primer was 5′-ATATTGAATTCTAGGCTAACCCAGACGGTCACCGTA-3′, and that for the reverse primer was 5′-TAATTAAGCTTAATCACTGTGACCGTGACGGG-3′, where the underlined sequence is from the merP gene and the sequence in bold is for the designed restriction sites EcoRI and HindIII, respectively. In lightface type are the flanking bases needed for the restriction enzymes to work effectively. The primers were designed to introduce an EcoRI site at the start of the merP gene and a HindIII site at the end. The PCR product was digested with EcoRI and HindIII and ligated into similarly digested pMAL-c2. The recombinant plasmid (pSSS) was transformed into competent DH5$\alpha$ E. coli cells. Successful transformants were screened by restriction digestion using a unique Nhel site within the merP gene. The DNA sequence was confirmed by the dyeoxide sequencing method (Sanger et al., 1977). Supercoiled plasmid was isolated from the DH5$\alpha$ strain and retransformed into BL21 E. coli cells which grow well in the minimal media used for isotopic labeling.

**Expression of MerP.** Uniformly 15N-labeled and 13C/15N-labeled merP were obtained by expression in minimal M9 media (11 g/L Na$_2$HPO$_4$·7H$_2$O, 3 g/L KH$_2$PO$_4$, 0.5 g/L sodium citrate, ~10 mg of thiamin, 1 mL of M MgSO$_4$·MgCl$_2$, 0.1 mL of CaCl$_2$) containing 1 g/L (15NH$_4$)$_2$SO$_4$ and 2 g/L [13C]$\delta$glucose (10 g/L unlabeled glucose) or by using 13C/15N-labeled algam media. A 1 mL culture in rich LB media was used to inoculate 25 mL of minimal media which was grown overnight. This was then used to inoculate 1 L of minimal media. The bacteria were grown to an OD$_{550}$ of about 0.5 before induction with IPTG (final concentration 0.5 mM). Incubation was continued for 2–3 h before being harvested by centrifugation. All cultures were maintained at 37 °C and contained 100 µg/mL ampicillin. Cells were immediately frozen and kept at −80 °C until further use.

**Purification of MerP.** Protein purification was monitored at each stage by Tris–tricine gel electrophoresis (von Schägger & Jagow, 1987). Where possible, all procedures were carried out at 4 °C. Cells from 1 L of growth media were resuspended in 20 mL of 20 mM sodium phosphate (pH 7.5) buffer containing 10 µg/mL lysozyme, 0.01% sodium azide, 50 mM dithiothreitol (DTT), and the protease inhibitors pepstatin A and leupeptin (1 µg/mL). For the preparation of merP in the reduced form, the buffer also contained 1 mM EDTA. The cell suspension was passed through a French press twice at 12 000 psi, and the resulting lysate was cleared by centrifugation at 50000g for 45 min at 4 °C. The supernatant was diluted to 100 mL with additional buffer and then passed over an amylose resin affinity column which bound the MBP-merP fusion protein. The column was washed with 3 volumes of 20 mM sodium phosphate (pH 7.5) to remove the unbound proteins. The fusion protein was eluted with sodium phosphate containing 10 mM maltose. All fractions containing fusion protein were combined and concentrated to ≥1 mg/mL (typically 50 mL/L of original growth media) using a YM30 membrane in an Amicon stirred cell. The fusion protein was cleaved with factor Xa at a concentration of 10 µg/mL for 1–2 days. After complete cleavage, DTT was immediately added to a concentration of 10 mM. The cleavage products were
structures of reduced and mercury-bound forms of merp

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Table 1: NMR Acquisition Parameters

<table>
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<tr>
<th>NMR experiments</th>
<th>nucleus</th>
<th>no. of points</th>
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<th>final matrix size</th>
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<td>120</td>
<td>48</td>
<td>2048</td>
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<tr>
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<td>HXNOE</td>
<td>15N, 13C</td>
<td>128</td>
<td>1024</td>
<td>35</td>
</tr>
</tbody>
</table>

Mercury-Bound MerP

|                |         | 128–256       | 2048               | 35               | 12             | 256–512       | var              |
| 15N-edited TOCSY | 1H, 15N| 120           | 48                 | 2048             | 12             | 35            | 12             | 256             | 128          | 896             | 24     |
| 15N-edited NOESY | 1H, 15N| 220           | 60                 | 2048             | 12.5           | 35            | 12.5          | 512             | 128          | 896             | 16     |
| NOESY          | 1H, 1H  | 896           | 2048               | 12               | 12             | 1024          | 2048            |
| TOCSY          | 1H, 1H  | 512           | 2048               | 12               | 12             | 1024          | 2048            |
| HDX           | 15N, 1H | 60            | 120                | 2048             | 35             | 12            | 128             | 128             | 442             | 24     |
| T<sub>1</sub>H| HSQC     | 15N, 13C      | 190                | 512              | 35             | 12            | 128             | 128             | 32          |
| T<sub>2</sub>H| HSQC     | 15N, 13C      | 190                | 512              | 35             | 12            | 128             | 128             | 32          |
| HXNOE          | 15N, 13C| 128           | 1024               | 35               | 12             | 256             | 1024             |

a Spectra were strip transformed in this dimension to include only the amide region (low field) part of the spectrum.

concentrated to 2 mL using a YM3 membrane and then applied to a Sephacryl S-100HR column (90 × 2.5 cm, Pharmacia, Piscataway, NJ) equilibrated with 20 mM sodium phosphate (pH 7.5) buffer, 0.01% sodium azide, 10 mM DTT, 1 mM EDTA (reduced samples only), and the protease inhibitors pepstatin A and leupeptin (1 µg/mL). As judged by silver-stained gel electrophoresis, the protein is very pure (95%). The solution containing merP was concentrated to 1–3 mM and dialyzed against the buffer used in the NMR samples.

Sample Preparation. Samples for NMR studies contained 1–3 mM protein in 20 mM phosphate buffer (pH 6.5) in 90% H<sub>2</sub>O/10% D<sub>2</sub>O or 100% D<sub>2</sub>O. Samples with the mercury-bound form of merP were prepared by carefully titrating Hg<sup>2+</sup> (typically HgCl<sub>2</sub>) into a previously prepared reduced merP sample until the NMR spectra stopped changing (approximately 1:1.5 [protein]:[Hg<sup>2+</sup>]). For 199Hg NMR samples, 199HgO was dissolved in concentrated phosphoric acid and then diluted to make a stock solution. After addition of a stock solution of 199Hg to the sample until the NMR spectra stopped changing (approximately 1:1.5 [protein]:[Hg<sup>2+</sup>]), the sample pH was readjusted to 6.5.

NMR Spectroscopy. All NMR spectra were recorded at 300 K on Bruker DMX500, DMX600, and DMX750 NMR spectrometers. NMR spectra were processed using XWIN-NMR (Bruker) or Felix ( Biosym) and analyzed using NMRCOMPASS (Molecular Simulations). All of the pulse sequences incorporated WATERGATE (Piotto et al., 1992), except where noted, for suppression of the water resonance. 15N decoupling was performed using the GARP sequence (Shaka et al., 1985). Mild exponential line broadening in the acquisition (1H) dimension (1–5 Hz) and a shifted (60–90°) squared sine bell in the indirect dimensions (15N, 13C) were applied to the free induction decays except where indicated.

Two-dimensional 1H–15N heteronuclear FHSQC (Mori et al., 1995) and HMQC (Bax et al., 1983) spectra were used to characterize the protein samples and as the starting point for the NMR studies. 15N resolved three-dimensional NOESY/HMOC experiments (Marion et al., 1989) were performed using mix times of 100, 125, and 250 ms. The 199Hg resolved three-dimensional TOCSY/HMOC spectra (Marion et al., 1989) were obtained with a mix time of 80 ms (MLEV) (Levitt, 1982) for both reduced and mercury-bound forms of merP. Two-dimensional 1H–1H NOESY and TOCSY spectra were obtained from samples of reduced and mercury-bound merP in D<sub>2</sub>O solution. The signal from residual HDO in the samples was suppressed with weak continuous irradiation of the water resonance during the recycle delay. NOESY spectra were acquired with mix times of 50 and 125 ms. TOCSY spectra were acquired with a mix time of 80 ms. 3H<sub>N,H</sub> coupling constants were measured with three-dimensional HNHA experiments (Vuisser & Bax, 1993). Water suppression was achieved with weak irradiation at the water resonance during the recycle delay. Backbone resonance assignments were made using the HNCA (Kay et al., 1990), HNCOCA (Bax & Ikura, 1991), and CBCA (Gresiek & Bax, 1992) three-dimensional triple-resonance experiments. WEX filter (Mori et al., 1994) experiments were performed using a two-dimensional HMOC version of the published pulse sequence with the WEX filter placed before the start of the HMOC pulse sequence. The second 1H 90° pulse of the WEX filter was substituted for the first 90° pulse of the HMOC sequence. Other parameters are described in Table 1. The mix times varied between 25 and 250 ms.

The one-dimensional 199Hg NMR spectrum was acquired using a direct-detect 10 mm broadband probe at 89.5 MHz on the DMX500 spectrometer. The sample volume was 1 mL, and the protein concentration was approximately 3 mM with an approximately 20% excess of 85.3%-enriched 199Hg<sup>2+</sup> in the solution. The spectrum was acquired with 450 000 scans in about 20 h with a sweep width of 200 kHz, a 20° pulse, and 150 ms recycle delay. It was processed with 1000 Hz of line broadening. The first few points of the FID were replaced by linear prediction to remove a
baseline roll in the spectrum. The spectrum was referenced with respect to neat dimethylmercury at 0 ppm.

$T_1$ and $T_2$ relaxation times were measured on the DMX600 spectrometer using two-dimensional $^1$H-detected $^{15}$N heteronuclear experiments (Kay et al., 1992) with the addition of gradient water suppression and gradient filters for artifact suppression. $T_1$ relaxation times were measured from ten two-dimensional data sets with relaxation delays of 10 ms, 50 ms, 100 ms, 200 ms, 300 ms, 500 ms, 600 ms, 800 ms, 1 s, and 1.5 s. $T_2$ relaxation times for mercury-bound merP were measured from six two-dimensional data sets with relaxation delays of 8, 16, 24, 40, 56, and 80 ms. Values for reduced merP were determined from seven data sets with relaxation delays of 8, 15, 22, 38, 45, 68, and 90 ms taken with 150 ($T_1$, $^1$H) × 1024 ($T_2$, $^1$H) points and 32 scans per increment. Processes in the $T_2$ ($^1$H) dimension was done with Gaussian multiplication for resolution enhancement to maximize the number of residues for which relaxation times could be measured.

Heteronuclear $^{1}$H-$^{15}$N NOEs were measured on a DMX600 spectrometer in the absence and presence of $^1$H saturation (Kay et al., 1989) which was accomplished with the application of 120° pulses at 20 ms intervals for 5 s before the first $^{15}$N pulse. Control experiments had a 5 s recycle delay to replace this $^1$H saturation period and ensure both experiments were the same length. To minimize the effects of chemical exchange between water and amide protons on the value of the measured NOE, experiments were performed with gradient water suppression and water flip-back techniques (Grzesiek & Bax, 1993b, Piotto et al., 1992). Experiments for both forms of merP were performed with 128 ($T_1$, $^1$H) × 1024 ($T_2$, $^1$H) points and 32 scans per increment.

Dynamics. All fitting of the experimental relaxation data was performed using the computer programs of Farrow et al. (1994). $T_1$ and $T_2$ values were determined by fitting the measured intensities of the peaks to a two-parameter equation for exponential decay. The steady-state NOE values were determined from the ratio of the intensities of the peaks with and without $^1$H saturation. The root mean square of the background noise from each experiment was used as an estimate of the standard deviation of the measured intensities.

A model-free formalism as expressed by eq 1 was used to fit the experimental data (Lipari & Szabo, 1982a,b)

$$J(\omega) = \frac{S^2 \tau_m}{1 + \omega^2 \tau_m^2} + \frac{(1 - S^2) \tau}{(1 + \omega^2 \tau^2)}$$

where the order parameter $S^2$ describes the degree of spatial restricted motion of the $^{1}$H-$^{15}$N bond vector and $\tau_m$ is the correlation time due to the tumbling of the whole molecule. The effective internal correlation time describing the rapid internal motions is described by $\tau_e$ in the equation:

$$\frac{1}{\tau} = \frac{1}{\tau_m} + \frac{1}{\tau_e}$$

In some cases an additional term, $R_{ex}$, as shown in eq 3 is needed to model transverse relaxation rates where there were additional contributions to relaxation, other than dipole–dipole interactions and chemical shift anisotropy, such as conformational exchange averaging

$$\frac{1}{T_2} = \frac{1}{T_{2(DD)}} + \frac{1}{T_{2(CSA)}} + R_{ex}$$

where the subscripts DD and CSA indicate the contributions from dipole–dipole and chemical shift anisotropy to transverse relaxation. Several variations of the function described by eq 3 were used to fit the experimental data: $\tau_e = 0$, $\tau_e$ included as a fitting parameter, $\tau_e = 0$ and including the term $R_{ex}$ to account for conformational exchange, and with both $\tau_e$ and $R_{ex}$ as fitting parameters.

NOE-Derived Restraints. NOE cross-peaks from the two- and three-dimensional NOESY experiments were classified as strong, medium, or weak corresponding to distance restraints of 1.9–2.7, 1.9–3.3, and 1.9–5.0 Å, respectively. Upper distance restraints involving nonstereospecifically assigned methylene, aromatic, and methyl protons were adjusted for center averaging (Wuthrich et al., 1983).

Torsion Angle Restraints. Torsion angle restraints for the $\phi$ angles were derived from an analysis of the $^3J_{\text{HN,Ha}}$ coupling constants measured from three-dimensional HNHA spectra. Restraints were included in the early rounds of calculations for the following coupling constants: $>8.5$ Hz ($-140 \pm 30^\circ$), $<5.5$ Hz ($-60 \pm 30^\circ$). Since some coupling constants can correspond to more than one torsion angle, restraints were only included for regular secondary structural elements as determined from the NOE data. During later stages of structure refinement if only one value of $\phi$ was consistent with the structure and the coupling constant, a corresponding restraint with large bounds ($\pm 50^\circ$) was included. In some cases the $\phi$ angle was simply restricted to negative values.

Hydrogen-Bonding Restraints. Slowly exchanging amide hydrogens were identified qualitatively from a series of two-dimensional $^1$H-$^{15}$N HMQC spectra recorded at various time intervals after a lyophilized uniformly $^{15}$N-labeled protein sample was dissolved in D$_2$O. Complementary information about rapidly exchanging hydrogens was obtained from a series of WEX-filtered HMQC spectra. Hydrogen bond restraints were introduced only after initial rounds of calculations revealed the fold of the protein. Distance restraints of 1.5–2.3 Å for $^1$H–O$_C$ and 2.4–3.3 Å for N$_{\text{H}}$–O$_C$ were added for secondary structural elements where the acceptor–donor pairs were unambiguous.

Structure Calculations. Structure calculations were performed using the computer program X-PLOR version 3.1 (Brünger, 1992). A hybrid distance geometry/simulated annealing protocol as described in the manual was used. The value of the NOE and torsion angle potentials were calculated with force constants of 50 kcal mol$^{-1}$ Å$^{-2}$ and 200 kcal mol$^{-1}$ rad$^{-2}$, respectively.

Initial calculations started from a linear polypeptide template with random backbone angles. An iterative approach similar to that described by Powers et al. (1993) was employed in later rounds of refinement. Additional experimental restraints were included as the quality of the structures improved and ambiguities in the NOE data were resolved. A simulated annealing refinement was repeated after each update of the NOE list. The final round of calculations was also started from a linear polypeptide template.

The abrupt distance cutoff ranges caused some NOEs that were close to the boundary of two ranges to be systematically violated. In such cases, these NOEs were reclassified into...
the next weaker class. As discussed by Clore et al. (1993), this improves accuracy at the expense of precision.

The five non-native residues at the N-terminus of the expressed protein were initially included in the calculations but were later omitted. They were not restrained by the experimental NMR data, and it was clear they did not form an integral part of the structural fold of merP in solution.

In the structure calculations for the mercury-bound form of merP the Hg–S dicoordinate bond length of 2.33 Å was specified (Utschig et al., 1993) with a force field of 500 kcal mol\(^{-1}\) Å\(^{-2}\). The force field for all other bonds was 1000 kcal mol\(^{-1}\) Å\(^{-2}\). The S–Hg–S bond angle was initially set to 180° with a force field of 70 kcal mol\(^{-1}\) rad\(^{-2}\), which is significantly lower than the 500 kcal mol\(^{-1}\) rad\(^{-2}\) used for all other bond angles.

RESULTS

Expressed MerP Protein. MerP prepared from the MBP fusion protein has five additional amino acids at the N-terminus. In order to avoid confusion, we utilize a numbering system that correlates directly with the wild-type amino acid sequence of merP. Residue 1 is Ala, and the extra residues that remain attached to the N-terminus after cleavage are designated -5 through -1. Amino acid analysis confirmed that the protein has the expected amino acid composition. Two-dimensional \(^{1}H\)–\(^{15}N\) HMQC spectra obtained from samples of uniformly \(^{15}N\)-labeled 77-residue merP isolated from the MBP fusion protein and 72-residue native merP isolated after expression from other vectors without the use of a fusion protein are nearly identical. Three of the five expected extra peaks are present in the spectrum of the fusion-derived protein, and the amide \(^{1}H\) and \(^{15}N\) chemical shift frequencies of the first few residues are slightly shifted. The extra resonances show no NOEs to residues other than those adjacent in the sequence, which suggests that they are not structured and do not interact with the rest of the protein.

Coordination of Metal. The coordination geometry of metal binding proteins can be probed by \(^{199}Hg\) NMR spectroscopy as demonstrated by O’Halloran and co-workers for several proteins, including the regulatory protein merR of the mer operon (Utschig et al., 1995). \(^{199}Hg\) NMR spectroscopy is ideal for studying the coordination geometry of merP since Hg\(^{2+}\) is its native metal and \(^{199}Hg\) is a spin \(S = \frac{1}{2}\) nucleus with a chemical shift range of more than 3000 ppm that is highly sensitive to its ligands. Figure 1 contains the directly detected one-dimensional \(^{199}Hg\) NMR spectrum of Hg(II) bound to merP in aqueous solution. The chemical shift of bound mercury is \(-816\) ppm, which is within the range observed for linear biocoordinate aliphatic thiolate compounds \([-816\) and \(-985\) ppm for Hg(S\(-n\)-Pr)\(_2\) and Hg-(SET)\(_2\), respectively] (Kubicki et al., 1981). In contrast, the \(^{199}Hg\) chemical shift observed for Hg(II) bound to merR which is tricoordinate (three Cys residues) is \(-106\) ppm (Utschig et al., 1995), and this result correlates well with those for structurally characterized tricoordinate aliphatic thiolate compounds. Thus, the chemical shift value of mercury bound to merP shown in Figure 1 demonstrates that the metal is bicoordinate and not tricoordinate.

Protein NMR Spectra. The two-dimensional \(^{1}H\)–\(^{15}N\) HMQC spectrum of uniformly \(^{15}N\)-labeled reduced merP in Figure 2A displays essentially complete resolution among all amide resonances. The line widths in both \(^{1}H\) and \(^{15}N\) frequency dimensions are narrow, and there is excellent chemical shift dispersion in both dimensions as well. Two-dimensional \(^{1}H\)–\(^{15}N\) HMQC spectra of the mercury-bound and reduced forms of merP are superimposed in Figure 2B. A number of amide resonances shift significantly when the protein binds mercury. Since the resonances from all backbone sites have been identified and assigned for both
forms of the protein (except for A15 in the mercury-bound form), lines are used to correlate the resonances most strongly affected by metal binding.

A combination of three-dimensional $^{15}$N-separated NOESY/HMQC and HOHAHA/HMQC (Marion et al., 1989) and triple-resonance experiments were used to sequentially assign resonances of the protein. HNCA spectra show a strong correlation peak from the amide hydrogen and nitrogen resonances to the interresidue $\alpha$-carbon resonance and a weak correlation peak to the $\alpha$-carbon resonance of the previous residue (Bax & Ikura, 1991), which provides the sequential residue to residue connection needed for assignments. However, some residues showed only one carbon peak due to a degenerate $^{13}$C chemical shift or the interresidue correlation being too weak to detect, and in these cases the HNCOCA or CBCACONH experiments were essential in order to complete the assignments. The CBCACONH and HNCOCA experiments gave strong correlations to C$\beta$ resonances in the previous residue, but the CBCACONH experiment provides the $\beta$-carbon chemical shift frequencies as well. Stretches of amide resonances were aligned sequentially by matching the C$\beta$ chemical shift frequencies as illustrated in Figure 3A. Ambiguities in the assignments could generally be resolved by referring to the NOE/HMQC data and looking for interresidue correlations, as shown in Figure 3B.

The C$\beta$ chemical shifts observed in the CBCACONH experiment were useful in identifying the most probable type of amino acid associated with each C$\alpha$ and C$\beta$ chemical shift (Grzesiek & Bax, 1993a). Gly residues have upfield chemical shifts and give rise to only one interresidue peak. Thr, Ser, and Ala residues also have distinctive C$\beta$/C$\gamma$ chemical shift pairs; for example, in Figure 3A the C$\beta$ chemical shift of T50 is downfield of the C$\gamma$ which is typical of Thr residues. All amino acid type assignments were compared to those predicted from the $^{15}$N resolved TOCSY/HMQC spectrum. In this way connected stretches of residues could be placed uniquely in the protein sequence. Breaks in the sequential connectivity occurred at prolines.

For the mercury-bound merP $^{15}$N resolved three-dimensional TOCSY/HMQC and NOESY/HMQC spectra were sufficient to make the sequential assignments, since only a subset of resonances had to be reassigned from those observed in the reduced form of merP.

Aromatic side chain $^1$H resonance assignments were determined from two-dimensional NOESY (50 and 125 ms mix times) and TOCSY (80 ms) experiments in D$_2$O solution. Connectivities were established in the standard way (Wüthrich, 1986) and then cross-checked with resonances observed in the $^{15}$N resolved three-dimensional experiments.

The experiments in D$_2$O solution were useful for obtaining critical side chain to side chain NOEs for helix–helix and sheet–helix contacts. Several resonances, in particular, V21 ($\gamma$-methyls), L57 ($\delta$-methyls), and L25 ($\delta$-methyls), are upfield shifted and well separated; these, and those from the aromatic residues F47 and Y66, provided many unambiguous NOEs essential for determining the overall protein fold. The upfield-shifted resonances also provided a convenient monitor of the binding of mercury to merP since they are single isolated peaks in the one-dimensional $^1$H NMR spectrum; for example, the methyl resonance from V21 shifts downfield when mercury binds to the protein.

Summaries of the short- and medium-range NOEs and other measurements for the reduced and mercury-bound forms of merP are shown in Figure 4. There are clearly two helices in merP, as indicated by the presence of $\alpha$N(i + 3) and $\alpha$N(i + 4) and strong NN(i + 1) NOEs. There are four regions of $\beta$ sheet, as indicated by strong $\alpha$N(i + 1) and only weak NN(i + 1). The chemical shift index data (Wishart et al., 1991, 1992) in Figure 4 agrees well with the secondary structure predictions from the NOE data with helical H$^\alpha$ chemical shifts below and $\beta$ sheet H$^\beta$ chemical shifts above those of a random coil polypeptide. The secondary structure elements at the top of Figure 4 are referred to as B1, H1, B2, B3, H2, and B4.

**Structure Calculations.** A total of 972 distance restraints were derived for the NMR experiments on the mercury-bound form of merP. Of these, 221 were interresidue, 323 sequential, and 428 medium and long range ($ij | ij \geq 2$). In addition, 67 torsion angle and 56 hydrogen bond restraints were used in the structure calculations. The total of 1095 corresponds to 15.2 restraints per residue. For the reduced form of merP 918 NOE distance restraints were used, consisting of 210 interresidue, 298 sequential, and 418 medium and long range as well as 61 dihedral and 56 hydrogen bond restraints. This is a total of 1035 restraints (14.4 per residue).

From the 80 structures that resulted from the final round of calculations for both mercury-bound and reduced forms of merP, 29 (mercury-bound) and 36 (reduced) contained no upper bound NOE violations greater than 0.5 Å or dihedral angle violations greater than 5°. The 20 lowest energy for each form were used for further analysis. Pertinent structural statistics are shown in Table 1. In general, all structures have good covalent geometry as shown by small deviations from idealized geometry. Ramachandran plots of $\phi, \psi$ angles are shown in Figure 5A,C for residues 3–71. The majority of residues are in energetically favorable regions. A few residues in loop regions have significantly positive values but still fall in energetically favorable regions of the Ramachandran plot.
Superpositions of the 20 lowest energy structures of both forms of merP are shown in Figure 6A,B. Nearly all of the polypeptide has a well-defined structure in both mercury-bound and reduced forms of merP, as indicated in the rmsd values listed in Table 2. The rmsd to the average for residues 3–71 is 0.361 and 0.355 Å for the reduced and mercury-bound forms of merP, respectively. If residues 10–18, which constitute the metal binding loop, are excluded, these values drop to 0.339 and 0.299 Å. If only those residues whose amide and nitrogen chemical shifts do not change significantly are included in the rmsd calculations (as indicated in Figures 5 and 6), these values drop even further to 0.322 and 0.270 Å. The spectra of the reduced form of the protein tend to have more chemical shift overlap, which limits the number of restraints that could be confidently extracted from the data. If residues 11–18 and 38–41, which form a turn near the metal binding site, are excluded from the Ramachandran plot as shown in Figure 5B,D, the differences between the two forms are minimal.

**Dynamics.** Comparisons of $T_1$, $T_2$, heteronuclear NOE, and the fitted order parameter $S^2$ for reduced and mercury-bound forms of merP are shown in Figure 7. The missing bar graph positions correspond to proline residues without amide resonances or other amino acids whose amide resonances partially overlap, making relaxation measurements unreliable. Residues 1, 2, and 72 are significantly mobile on a fast time scale as indicated by the low values of the order parameter $S^2$, the lower values of the heteronuclear NOEs, and the higher values of $T_1$ and $T_2$. In addition, the residues in the loop connecting strand B3 and the second helix (residues 51–53) show some evidence of mobility. This is most obvious in the values of $T_1$ (Figure 7E,F), which are significantly longer than for the majority of residues in the protein. The largest differences, however, between the two forms of the proteins are in the values of $T_2$ observed for residues 11–18 in the metal binding loop. The $T_2$ values for these residues in the reduced form of the protein are shorter than those observed in the mercury-bound form. In contrast, there are no apparent differences in the values of $T_1$ or NOE observed for these residues. This resulted in the need for an exchange term, $R_{ex}$, in the fitting of the order parameter for residues 11–18. The value of the exchange term is included in Figure 7A,B where it provided the best fit of all considered models described in the Materials and Methods section.
Description of the Structure. MOLSCRIPT (Kraulis, 1991) representations of the structures of the reduced and mercury-bound forms of merP are shown in panels C and D of Figure 6, respectively. The two R helices are formed by residues 19-27 for mercury-bound merP (17-27 in reduced form) and residues 54-64 in both forms. The two helices lie above the plane of a four-strand $\beta$ sheet formed by residues 3-9, 31-37, 42-47, and 69-70. The four-strand $\beta$ sheet has a slight left-handed twist common in many antiparallel $\beta$ sheets. This twist is more pronounced in the last short strand. A $\beta$ bulge between residues S32, K33, and T46 breaks the sheet at the beginning of strand B2. The helices are roughly parallel and are oriented at an angle of about 15° relative to the axis of the $\beta$ sheet. This twist is more pronounced in the last short strand. A $\beta$ bulge between residues S32, K33, and T46 breaks the sheet at the beginning of strand B2. The helices are roughly parallel and are oriented at an angle of about 15° relative to the axis of the $\beta$ sheet. The interhelical angle is about 50°. This follows the twist of the $\beta$ sheet as the helices are packed against the sheet. The long loop connecting strand B1 and the first helix contains the metal binding site with the GMTCAAC sequence. In the mercury-bound form the two Cys residues lie above the loop toward the surface of the protein. The S-Hg-S bond angle 177° is close to linear.

Chemical Shift Differences. Figure 2B contains the two-dimensional $^1$H-15N HSQC spectra of reduced and mercury-bound forms of merP. There are dramatic differences in the chemical shifts of a few amide resonances. The chemical shift differences for the amide $^1$H and $^{15}$N and $^1$H$^\beta$ resonances are plotted in Figure 8. Significant resonance shifts are localized to three main regions of the protein. The largest changes occur in the loop connecting $\beta$ sheet strand B1 and helix H1 and part of the way into helix H1. This is the region...
that contains the GMTCAAC binding site of the mercury. Smaller changes occur in the region between strands B2 and B3. This turns out to be directly below the proposed binding loop in the three-dimensional fold. The final region of shift change is smaller, but still significant. This is the turn connecting helix H2 and strand B4. Again, this is in the vicinity of the binding loop in the three-dimensional structure. Figure 9 contains a representation of merP colored to show the regions with the largest chemical shift differences between the two forms of merP.

DISCUSSION

Structure of MerP. Both the reduced and mercury-bound forms of merP have essentially the same global fold, which consists of two antiparallel helices overlaying a four-strand $\beta$ sheet. The secondary structure and overall fold derived from earlier homonuclear NMR experiments on the oxidized form of merP (Eriksson & Sahlman, 1993) are consistent with the three-dimensional structures of the reduced and mercury-bound forms of merP shown in Figure 6. The SCOP database (Murzin et al., 1995) indicates that the $\beta$$\alpha$$\beta$$\alpha$$\beta$ fold defines a class of $\alpha$$\beta$ proteins with antiparallel $\beta$ sheets and segregated $\alpha$ and $\beta$ sections. The fold can be described as an $\alpha$$\beta$ sandwich characteristic of “ferredoxin-like” proteins which have diverse functions. This class of proteins includes small proteins and domains of larger proteins such as RNA binding protein domains, DNA binding protein domains, acyl phosphatases, and the phosphoglycerate dehydrogenase regulatory domain. They do not appear to have a functional theme in common with other heavy metal binding proteins, and the active sites to these proteins vary dramatically. For example, one of the largest groups of protein that gave this fold is the family of RNA binding proteins. Even among members of this family of proteins there is considerable functional diversity. The binding site of these proteins is usually localized to the outer surface of the $\beta$ sheet (Bird & Dreyfuss, 1994). This is very different from the situation found for merP.

The spectroscopic differences between the two forms of the protein, as highlighted in Figure 9, are localized near the GMTCAAC-containing loop connecting the first strand of the sheet to the first helix. They involve residues distant in the primary sequence but close in the folded structure including those near the turns including residues 38–41 and 64–66. The finding of changes in the fluorescence of Y66 upon binding Hg(II) is consistent with this (Summers, personal communication). The structural representations in Figure 10 allow detailed comparisons of the metal binding loop in the two forms of the protein. The two Cys residues are much further away from each other in the reduced form than in the mercury-bound form. Binding of mercury causes a slight unwinding of the helix as the two cysteines become closer. Another difference is the position of the aromatic residue F38 which lies below the metal binding loop. In the reduced form the side chain is oriented toward the binding loop. The two-dimensional homonuclear NOE spectrum of the reduced form of merP has more than eight NOE cross-peaks between Phe ring resonances and those from residues 9 through 17. In contrast, the aromatic ring moves closer to the surface of the protein in the mercury-bound form, and no NOE cross-peaks are observable from the same Phe ring hydrogens. The movement of this aromatic ring may account for the dramatic $^{15}$N and $^1$H chemical shift differences for some of the backbone resonances in the two forms of the protein.

The S–Hg–S bond is approximately linear in the structure of the mercury-bound form of merP, and this is confirmed by the $^{199}$Hg chemical shift of the bound mercury ion. The coordination geometries of many model compounds have been examined (Utschig et al., 1993; Wright et al., 1990). The most common primary coordination number for Hg(II) is 2. These compounds have covalent bond lengths that vary between 2.316 and 2.361 Å and bond angles that vary between 180.0° and 167.4° (Wright et al., 1990). Distortions from linearity are generally caused by longer secondary bonding interactions found in the solid state. MerP clearly binds Hg(II) with two Cys ligands. This is in agreement with mutagenesis that show both Cys residues to be essential for specific mercury binding to the protein (Sahlman & Skärfstad, 1993). The slight deviation of the S–Hg–S bound linearity may be caused by an interaction with a water molecule. The binding site of merP is very different from that of merR, which has a tricoordinate binding site (Utschig et al., 1993).
Another difference between the two forms of merP is their dynamics. An increase in $T_1$ or $T_2$ for some resonances compared to those from the bulk of the protein generally suggests that the residues undergo some extra modes of rapid motion. However, when there is a drop in $T_1$, with no related drop in $T_2$, this does not suggest there is less motion but rather that the motion is on a different time scale. The greater number of exchange terms needed to fit the order parameter $S^2$ for the reduced form of the protein suggests that the binding loop may undergo an additional motion on a slow time scale. There does not appear to be any evidence of multiple conformations in the NOE data, as there are fewer NOEs per residue compared to the bulk of the protein through this binding loop for both forms of the protein. This is partly due to the exposed and extended nature of this loop. Although the loop as a whole has a single conformation, it may “flip” or “breath” slowly without a bound metal. When the Cys residues interact with the mercury, they stabilize the entire loop, giving it a very well-defined conformation.

The primary role of merP appears to be that of a scavenger of free Hg(II) in the cell periplasm (Silver & Walderhaug, 1995). After binding Hg(II), merP passes it to the next protein in the chain, merT, which transports it across the cell membrane into the cytoplasm where it is reduced by the enzyme mercuric reductase to Hg(0). In this context, it is not surprising that major structural changes are not observed when merP binds mercury. A global structural rearrangement could be counterproductive, since it might move the mercury ion away from the surface of merP to a place where it would be inaccessible to merT. Nonetheless, the metal binding loop does appear to have some conformational flexibility, and a transient intermediate conformation may be involved in passing Hg(II) to merT from merP. The “bucket brigade” mechanism, suggested as a way of passing the mercury from protein to protein in the detoxification pathway (Brown et al., 1991), would be consistent with the mercury from protein to protein in the detoxification “bucket brigade” mechanism, suggested as a way of passing.

REFERENCES

Structures of Reduced and Mercury-Bound Forms of MerP

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