Reactivities of the two essential cysteine residues in the heavy metal binding motif, MTC\textsubscript{14AAC17}, of the periplasmic Hg\textsuperscript{2+}-binding protein, MerP, have been examined. While Cys-14 and Cys-17 have previously been shown to be Hg\textsuperscript{2+}-binding residues, MerP is readily isolated in an inactive Cys-14–Cys-17 disulfide form. In vivo results demonstrated that these cysteine residues are reduced in the periplasm of Hg\textsuperscript{2+}-resistant Escherichia coli. Denaturation and redox equilibrium studies revealed that reduced MerP is thermodynamically favored over the oxidized form. The relative stability of reduced MerP appears to be related to the lowered thiol pK\textsubscript{a} (5.5) of the Cys-17 side chain. Despite its much lower pK\textsubscript{a}, the Cys-17 thiol is far less accessible than Cys-14, reacting 45 times more slowly with iodoacetamide at pH 7.5. This is reminiscent of proteins such as thioredoxin and DsbA, which contain a similar C-X-X-C motif, except in those cases the more exposed thiol has the lowered pK\textsubscript{a}. In terms of MerP function, electrostatic attraction between Hg\textsuperscript{2+} and the buried Cys-17 thiolate may be important for triggering the structural change that MerP has been reported to undergo upon Hg\textsuperscript{2+} binding. Control of cysteine residue reactivity in heavy metal binding motifs may generally be important in influencing specific metal-binding properties of proteins containing them.

Bacterial resistance to mercuric ion is mediated by the polypeptides encoded by mer operons. Although the specific number of required polypeptides varies according to species, all operons appear to encode: one or more regulatory proteins (MerR, MerD); a periplasmic mercuric ion-binding protein (MerP); one or more integral membrane proteins thought to be required for mercuric ion transport (MerT, MerC); and a cytoplasmic mercuric ion reductase (MerA), which reduces intracellular Hg\textsuperscript{2+} to a volatile form, Hg\textsuperscript{0} (reviewed in Refs. 1 and 2).

The mer operon-encoded periplasmic protein, MerP, from the transposon Tn21, has a molecular mass of 7500 Da after removal of a periplasmic signal sequence (1, 3, 4). There are no tryptophan or histidine residues in the mature protein and only two cysteine residues, at positions 14 and 17. MerP has previously been shown to specifically bind Hg\textsuperscript{2+} in the presence of external thiol via these two cysteine residues (4, 5). The recently published structure of MerP revealed that Cys-14 is surface-exposed and Cys-17 is buried inside the protein; once Hg\textsuperscript{2+} is bound to the thiol, they are both surface-exposed (6). The structural change that accompanies Hg\textsuperscript{2+} binding has been proposed to be important for the interaction of Hg\textsuperscript{2+}-loaded MerP with the inner membrane transport protein(s). Although it has been postulated that the function of MerP is to transfer Hg\textsuperscript{2+} to the mer operon-encoded integral membrane proteins for passage across the inner membrane (7), current evidence suggests that MerP is dispensable for transport. Instead, it may function as a mercuric ion “spoon” to protect components of the periplasm from the toxic effects of this heavy metal (8, 9).

MerP has been shown to exist in vitro in oxidized (disulfide) and reduced (dithiol) forms, but the redox state of the protein in the bacterial periplasm has never been reported. In order to bind Hg\textsuperscript{2+} via its thiol, MerP should be present in the reduced form. However, it is generally accepted that the periplasm is an oxidizing environment, with proteins such as DsbA and DsbB catalyzing disulfide bond formation in many bacterial periplasmic proteins (reviewed in Ref. 10). In this investigation, the redox state in vivo and the reactivity of the thiol groups of MerP have been investigated.

MATERIALS AND METHODS

Chemicals—All chemicals were of the highest purity available. \textsuperscript{209}HgCl\textsubscript{2} was purchased from Amersham Pharmacia Biotech (Stockholm, Sweden).

Bacterial Strains and Manipulations—The gene, merP, from transposon Tn21, was expressed in E. coli BL21(DE3) (11) from the T7 promoter of pCA (4), or using the plasmid pDU1003, which contains the complete Hg\textsuperscript{2+}-inducible mer operon (12). Expression of the MerP variants C14A, C14S, C17A, and C17S was as described previously (5). The C17D variant was constructed using the QuikChange method (Stratagene). The template was pCA\textsubscript{MerP}, and the mutagenic primers were 5’-GACTTGGCCGCGGACCGATCACAGTC-3’ and its complement, obtained from Biocorp Inc. (Montreal, Canada). Nucleotide sequence analysis of the complete gene was performed at the Sheldon Biotechnology Center (Montreal, Canada), and confirmed the presence of the desired mutation.

In all cases, cultures were grown in LB medium, plus the appropriate antibiotic, to an A\textsubscript{max} of 0.8–1.0 before induction. Induction from T7-based plasmids was achieved by adding isopropyl-1-thio-\(\beta\)-D-galactopyranoside to a concentration of 0.5 mM, followed by further growth for 3 h at 37 °C. For induction from pDU1003, HgCl\textsubscript{2} was added to an initial concentration of 20 \(\mu\)M, and a second aliquot (20 \(\mu\)M) was added after an additional 1 h of growth at 37 °C.

Release of periplasmic proteins was achieved either using chloroform (13) or lysozyme-EDTA (14). In order to examine the redox state of MerP, iodoacetate was included to trap free thiols at all stages of the release procedure (14). Alternatively, cultures were processed by precipitation of cellular proteins using trichloroacetic acid, followed by dissolution of the precipitated proteins in 8 M urea, containing iodoacetate, essentially as described (15).

Proteins and Protein Modification—MerP and all of the variants were purified in the absence of added cysteine, as described previously; chromatography on hydroxylapatite was usually included as a final step.
(4, 5). Since all of these proteins were from recombinant sources never exposed to mercu- rial ion, residual bound Hg$^{2+}$ is not a concern in any experiments done with purified MerP or variants.

When required, wild-type MerP was reduced either using dithiothreitol (DTT) or tris-(2-carboxyethyl)phosphine (TCEP), under conditions described in the text. Modification of MerP by iodoacetamide was accomplished at room temperature in the presence of sodium iodoacetate (0.1 M) in 0.1 M Tris-HCl, pH 8.1. The modification reactions were generally run in the dark for 10–30 min, and then placed on ice before analysis. In some cases, urea was included during the modification reaction.

Protein concentrations were estimated by the method of Gill and von Hippel (16).

Thiol Group Titration—The pK$a$ values of cysteine thiol groups were monitored by the change in absorbance at 240 nm accompanying ionization (17). Over the pH range tested, the oxidized form of MerP exhibited no absorbance change at 240 nm, indicating that the changes observed during pH titration were due to thiol group ionization. In preparation for these experiments, samples were reduced at room temperature for 1–5 h with a 10-fold excess of TCEP. Excess reductant was removed by gel filtration on Econo-Pac10DG columns (Bio-Rad) equilibrated with 10 mM acetate buffer, pH 5.0, containing 0.2 mM EDTA. Samples were then concentrated using Centricron-3 (Amicon) ultraltration. Absorbance measurements were made by diluting a 30–50-fold concentrated solution of protein into 0.1 M acetate-MES-Tris buffers of varying pH (18) containing 0.1 M KCl. Water was added to give final buffer and KCl concentrations of 0.05 M each, and protein concentrations in the range of 50–120 μM. Spectra for each sample, contained in a microcell, were scanned immediately in the region 220–400 nm. A separate base line was recorded for each buffer prior to scanning the protein solution.

Thiol pK$a$ values were estimated by non-linear least-squares fitting of plots of extinction (240 nm) versus pH using equations for 1 or 2 pK$a$ values as supplied with Graft 3 (Erthasic Software, Middlesex, United Kingdom).

Determination of Redox Potential—The redox equilibrium constant of MerP with glutathione, $K_{ox}$, is shown in Equation 1.

$$K_{ox} = \frac{[\text{MerP}]}{[\text{GSH}]/[\text{GSSG}]} \frac{[\text{MerP}]}{[\text{GSH}]/[\text{GSSG}]}$$

(Eq. 1)

GSH and GSSG are the oxidized and reduced forms of glutathione, respectively. $K_{ox}$ was estimated after incubation of MerP with various ratios of oxidized and reduced glutathione, as described elsewhere (19). Briefly, MerP$_{red}$ (90 μM) was incubated anaerobiocally for 24 h in 0.1 M potassium phosphate buffer, pH 7.5, containing EDTA (1 mM), KCl (0.1 M), GSH (10.4 mM), and various concentrations of GSSG. Oxidized and reduced forms of MerP were quantitated by HPLC (see below) after quenching to pH 2 with HCl.

Data were fitted to Equation 2 (19, 20).

Fraction reduced = $R/GSH + [R/GSH] + K_{ox}/[GSH] + K_{ox}$

(Eq. 2)

where $R$ = [GSH]/[GSSG] or $K_{ox}/[GSH]$ is the equilibrium constant between glutathione and the MerP-glutathione mixed disulfide. Only small quantities (≤10%) of mixed disulfide accumulated, and a $K_{ox}$ of 0.56 was estimated from a plot of [MerP-GSG]/[MerP]$_{red}$ versus [GSSG]/[GSH], as described in Ref. 21.

The standard redox potential was calculated using the Nernst equation and a standard redox potential of +0.24 V (22) for the glutathione redox pair.

Quantitation of Oxidized, Reduced, and Mixed Disulfides of MerP—Different forms of MerP were quantitated using HPLC. The reduced, oxidized, and modified forms of the protein were separated on a Vydac C-18 Protein and Peptide column (catalog no. 218TP54) at a flow rate of 1.5 ml/min. Samples were acidified to a pH of approximately 2 with 15% acetonitrile, 0.05% trifluoroacetic acid, and after washing the column, it was allowed to be determined; corrections for nonspecific binding of 203Hg to the upper and lower chambers allowed the concentration of MerP-bound Hg$^{2+}$ to be determined; corrections for nonspecific binding to the concentrator were determined for each HgCl$_2$ concentration by identical ultrafiltration experiments carried out in the absence of MerP (4).

Data from two separate experiments were analyzed using Graft with non-linear least-squares fitting to the following equation to estimate the capacity for binding Hg$^{2+}$ and the apparent dissociation constant ($K_d$).

$$y = \frac{(K_d + t + C) - [(K_d + t + C)^2 - (4 \times t \times C)]^{0.5}}{2}$$

(Eq. 4)

$y$ is the concentration of bound Hg$^{2+}$, and $t$ the total concentration of added Hg$^{2+}$. This equation uses total added Hg$^{2+}$, rather than the amount of free versus bound, since the presence of cysteine in the assay makes it difficult to estimate the concentration of free Hg$^{2+}$.

RESULTS

Electrophoretic Methods for Detecting Oxidized and Reduced Forms of MerP—The in vivo oxidation state of MerP has never been determined.
been reported, although only the reduced form can bind Hg\(^{2+}\) via Cys-14 and Cys-17 (4–6). These cysteine residues can form a disulfide bond since reduced (MerP\(_{\text{red}}\)) and oxidized (MerP\(_{\text{ox}}\)) forms have been obtained by isolation of the protein in the presence or absence of cysteine in purification buffers (4). As is shown in Fig. 1, purified MerP\(_{\text{red}}\) (lane 1) and MerP\(_{\text{ox}}\) (lane 4) have different mobilities on a native gel; the reduced form migrates more slowly toward the cathode (bottom) in this gel system, possibly because of thiol group ionization at neutral pH (see below). However, in order to preserve and identify MerP\(_{\text{red}}\) in periplasmic extracts, it is necessary to derivatize the free thiols prior to electrophoresis.

Reaction with iodoacetate or iodoacetamide to trap reduced thiols and produce proteins with altered electrophoretic mobility has been used for a number of other thiol-containing redox proteins (see, e.g., Refs. 15 and 21). Reaction of MerP\(_{\text{red}}\) with iodoacetamide resulted in the conversion of the reduced form to a species that migrated at the position of the oxidized protein, as would be expected by blockage of an ionized thiol group(s) with the uncharged acetamide group (data not shown). On the other hand, reaction of reduced MerP with iodoacetate resulted in production of two slower migrating forms (Fig. 1, lane 3). Only the most slowly migrating form was observed over longer periods of reaction time (data not shown), or when MerP was reacted with iodoacetate in the presence of urea (Fig. 1, lane 2). Therefore, it appears that the two thiols on the protein are modified at quite different rates. The observed change in mobility is consistent with the expected addition of negative charge to MerP upon carboxymethylation. No change in the mobility of oxidized MerP was observed in the presence of iodoacetate, as would be expected if only the protein thiols were reacting (data not shown).

The identities of the faster and slower migrating carboxymethylated forms were examined using electrospray mass spectrometry. A sample prepared in the presence of urea and showing only the upper band on a native gel exhibited a single major peak with a molecular mass of 7,590; this molecular mass corresponds to that of doubly carboxymethylated MerP (7,472 (observed) + (59 \times 2)). In a sample containing mostly the faster migrating carboxymethylated form a species corresponding to singly carboxymethylated MerP (M\(_r\) = 7531) was the major product, and the doubly carboxymethylated form was the minor product.

In the next section, results are described using iodoacetate to trap and identify the reduced form of MerP from the periplasm of whole cells. The initial observations suggesting differing accessibility and p\(_K_a\) values of the two thiols will be addressed further in later sections.

**Redox State of MerP in Vivo**—In these experiments, cultures were exposed to iodoacetate to trap the reduced form of MerP, which was then released from the periplasm in the presence of iodoacetate and analyzed using polyacrylamide gel electrophoresis. Similar methods have been used for other periplasmic thiol-containing proteins (see, e.g., Refs. 14 and 30). When MerP was expressed alone from the T7 promoter, samples taken at various times throughout the induction period were all mostly in the oxidized form (Fig. 2A). Similar results were obtained when HgCl\(_2\) (20 \(\mu\)M) was added together with the inducer (data not shown), indicating that the presence of Hg\(^{2+}\) is not sufficient to maintain the reduced form. However, MerP was mainly in the reduced form in periplasmic extracts from Hg\(^{2+}\)-resistant cells harboring the complete operon (Fig. 2B, far left lane). In this strain, MerP remained reduced for up to 90 min after Hg\(^{2+}\) had been removed from the culture (Fig. 2B). Similar results for each strain were obtained using an alternative method (15) in which cellular proteins were precipitated with trichloroacetic acid to prevent possible nonspecific redox reactions after cell disruption (results not shown). It should be noted that the levels of MerP expression in the strains used for the experiments shown in Fig. 2 are comparable, despite the differences in the plasmid constructs. From these experiments it can be concluded that MerP is in the reduced form in the periplasm of Hg\(^{2+}\)-resistant cells, and that one or more of the mer operon-encoded proteins may be involved in keeping its thiols reduced.

**Redox Properties of Purified MerP**—The apparent stability of the reduced form of MerP in the oxidizing environment of the periplasm prompted us to examine the redox potential of the protein. Oxidized MerP was incubated anaerobically with various ratios of oxidized and reduced glutathione, and the ratios of oxidized and reduced MerP were estimated using reverse-phase HPLC after acid quench (Fig. 3). The equilibrium constant (K\(_e\)) with glutathione was estimated to be 27 mm, which corresponds to a redox potential of \(-190\) mV. This value is approximately midway between the redox potentials of oxidizing proteins such as DsbA (approximately \(-100\) mV) (21, 31), and reductants such as thioredoxin \((-270\) mV) (32).

**Thiol Group pK\(_a\) Values**—The reactivities of the two thiol groups in MerP should be governed by their respective p\(_K_a\) values, since the ionized thiol is more reactive than the protonated form (33). Native gel electrophoresis results (see above) suggested that at least one of the two thiol groups is ionized at neutral pH. The mobilities of single cysteine variants of MerP were thus examined using the native gel electrophoresis system, both before and after reaction with iodoacetate (Fig. 4) or...
iodoacetamide (data not shown). Since mass spectrometry indicated modification of some variants by cysteine, or dimerization (data not shown), all samples were reduced using DTT or TCEP prior to electrophoresis or modification. The majority of the reduced C14S sample ran at the position of the oxidized native MerP (Fig. 4, lane 2). Thus, it appears that it is Cys-17 which is ionized at the pH of the gel (gel buffer pH = 7.0). Consistent with this, C17D, unlike C17S, migrates at the position of reduced MerP (Fig. 4, lane 3).

The electrophoretic mobilities of unmodified proteins can be compared with the mobilities of carboxymethylated samples (Fig. 4, lanes 4–6). The carboxymethylated and non-carboxymethylated forms of C14S have the same mobilities (Fig. 4, lanes 1 and 4), as would be expected if Cys-17 is already ionized. Carboxymethylation of Cys-14 in C14S results in a negatively charged carboxymethylated derivative. Finally, carboxymethylated C17D migrates at the same position as the negatively charged carboxymethylated derivative. These observations add support for the conclusion that Cys-17 is ionized at neutral pH, whereas Cys-14 is not.

Since MerP has only two thiol groups, estimation of the \( pK_a \) values is possible by pH titration and monitoring the appearance of the thiolate forms at 240 nm (\( \epsilon \approx 4,000 \text{ M}^{-1} \text{ cm}^{-1} \)) (17). Controls using oxidized MerP and variants in which each thiol had been replaced by alanine or serine allowed specific contributions of each thiol group to the absorbance changes to be assessed. Representative results of these titrations are shown in Fig. 5, and \( pK_a \) values are summarized in Table I. The native reduced protein showed two ionizations with \( pK_a \) values of 5.5 and 9.16 (Fig. 5A); oxidized MerP showed no appreciable change in absorbance over this pH range, indicating that ionization of the single tyrosine residue does not contribute to the observed absorbance changes (data not shown). The C14A and C14S variants showed single absorbance changes titrating with \( pK_a \) values of 6.08 and 5.6, respectively, while the C17A and C17S variants also revealed single thiol ionizations with \( pK_a \) values of 7.7 and 7.4, respectively (Table I and Fig. 5B). Thus, it appears that the thiol with a \( pK_a \) of 5.5 in native MerP is Cys-17, while that with a \( pK_a \) of 9.2 is Cys-14, although the \( pK_a \) of this thiol appears to be considerably perturbed in the C17A and C17S variants.

A possible reason for the lowered \( pK_a \) values of Cys-14 in the C17A and C17S variants is the absence of a negative charge at residue 17 at neutral pH. Results obtained for the C17D variant showed a single thiol titrating with a \( pK_a \) of 9.1 (Fig. 5B), confirming the conclusion that it is Cys-14 which has this \( pK_a \), and suggesting that the presence of a negative charge at position 17 is necessary to maintain the native-like conformation around Cys-14 in reduced MerP. In turn, this could affect the \( pK_a \) of Cys-14.

Kinetics of MerP Thiol Reaction with Iodoacetamide—In the initial electrophoretic analysis of modified MerP (Fig. 1), it was apparent that the two thiol groups reacted with iodoacetate at quite different rates. This observation is consistent with the NMR structure of reduced MerP, which shows that Cys-14 is on the surface of the protein while Cys-17 is buried (6). However, in addition to accessibility, the reactivities of the thiol will also depend on the fraction in the thiolate form. Since Cys-17 has a much lower \( pK_a \) than Cys-14, it was of interest to determine which of the two thiols reacts rapidly with a small thiol reagent such as iodoacetamide.

The kinetics of reaction of C17D and C14S with iodoacet-
amide are shown in Fig. 6. From these data, it is clear that Cys-14 reacts much more rapidly ($k_{app} = 0.95 \text{ M}^{-1} \text{s}^{-1}$, Fig. 6B) with this small neutral thiol reagent than Cys-17 ($k_{app} = 0.021 \text{ M}^{-1} \text{s}^{-1}$, Fig. 6A), which is fully ionized at the pH of the experiment. Comparable apparent second order rate constants of 1.5 and 0.029 $\text{M}^{-1} \text{s}^{-1}$ were observed for conversion of MerP$_{red}$ first to singly and then to doubly modified protein. Correcting for the percentage of each thiol in the anion form to obtain $k_s$, the pH-independent rate constant for reaction of the thiolate form, gave a value for Cys-14 that is 1800 times higher than that for Cys-17 in the single cysteine variants. This indicates that Cys-17 is sterically inaccessible and very slow to interact with external reagents despite the fact that, at pH 7.5, it is in the very reactive thiolate form and Cys-14 is not.

**Stabilities of Oxidized, Reduced, and Variant MerP**—The influence of structural differences between MerP$_{ox}$ and MerP$_{red}$ on protein stability were probed using guanidinium hydrochloride denaturation studies (Fig. 7). The thermodynamic parameters obtained assuming a two-state transition are summarized in Table I. The thermodynamic parameters obtained for denaturation of MerP$_{ox}$ are in excellent agreement with those reported previously (26). Changes in free energy of unfolding, $\Delta \Delta G^\circ$, were calculated by multiplying the average m-value for the two forms of MerP by the difference in transition midpoint ($\text{[guanidinium hydrochloride]}_{1/2}$) between the variant and wild-type proteins (34). MerP$_{red}$ unfolded with a guanidinium hydrochloride$_{1/2}$ significantly higher than that observed for the oxidized form (Fig. 7), with a $\Delta \Delta G^\circ$ of 0.85 kcal/mol. MerP$_{red}$ is thus thermodynamically more stable than MerP$_{ox}$.

The variant MerPs were generally less stable than wild-type MerP$_{red}$ (Table I). The C17A and C17S variants were, like MerP$_{ox}$, 0.7–0.8 kcal/mol less stable than MerP$_{red}$, but the C17D variant was not destabilized relative to MerP$_{red}$. These results support the notion that the negative charge at position 17 is important in maintaining a conformation more like the reduced than the oxidized form of MerP. Also consistent with this, the stability of C14S, where ionized Cys-17 is present, is almost identical to that of MerP$_{red}$. However, the stability of C14A is anomalously low, suggesting that the hydrophobic alanine residue is not well tolerated at position 14.

**Binding of Hg$_2^{+}$ at Low pH**—The finding that one of the MerP thiol groups is ionized at neutral pH prompted us to examine whether Hg$_2^{+}$ binding is affected when this thiol group is protonated. Titration of reduced MerP (9 $\mu M$) with Hg$_2^{+}$ was carried out in the presence of external cysteine, as described previously (4) except at pH 4 (Fig. 8). The apparent $K_d$ was 4.7 ± 1.9 $\mu M$, with a total binding capacity of 6.0 $\mu M$ ± 0.6 $\mu M$ (0.7 mol/mol protein). These results are similar to those reported for pH 7.3, where the apparent $K_d$ and binding capacity using 10 $\mu M$ MerP were 3.7 ± 1.3 and 8.8 ± 0.6 $\mu M$ (0.88 mol/mol protein), respectively (4).

**DISCUSSION**

The role of periplasmic MerP in mercuric ion resistance is postulated to be scavenging of mercuric ion via the heavy metal binding motif, GMTC$_{14}$XXC$_{17}$, for later transfer to Hg$_2^{+}$-translocating membrane proteins. Consistent with this role, MerP with both Cys-14 and Cys-17 reduced has been shown to bind mercuric ion with high affinity, even in the presence of excess free cysteine (4–6). Since MerP is readily isolated in the oxidized form, with a disulfide formed between Cys-14 and Cys-17, the reactivity of these thiol groups is an important determinant of the Hg$_2^{+}$-binding role of MerP.

Since various redox catalysts (the Dsb proteins) active with protein thiols/disulfides are present in the bacterial periplasm (reviewed in Ref. 10), a relevant question is whether MerP in vivo exists in the dithiol, Hg$_2^{+}$-binding form. Trapping experiments established that in the absence of expression of the other proteins of the mer operon, periplasmic MerP was mainly oxidized, while in cells expressing the complete operon, MerP was mainly in the reduced form. Thus, in mercuric ion-resistant cells, periplasmic MerP indeed exists in the Hg$_2^{+}$-binding form. The observation that MerP was mainly in the oxidized form when expressed alone suggests the possibility that association with other mer operon proteins is important to preserve the reduced form, but this has not been confirmed.

The reduced form of MerP was found to be thermodynamically more stable than the oxidized form. While disulfide bonds in proteins are generally considered to be a stabilizing influence, in some cases they are destabilizing. A well known exam-
ple of a destabilizing disulfide bond is the one found in DsbA (21). Unfolding experiments showed stabilization of MerP\textsubscript{red} relative to the oxidized form, with \(\Delta G_{50\%} = 0.85\ \text{kcal/mol}\). Unfolded MerP\textsubscript{ox} is theoretically 1.86 kcal/mol less stable than the unfolded reduced form, assuming that the only difference between the unfolded forms (35) is the reduced entropy associated with the 4-amino acid loop present in MerP\textsubscript{ox}. Taking this into account, the overall difference in free energy between the reduced and oxidized forms of MerP is calculated to be about 2.7 kcal/mol.

A number of factors appear to be important for stabilizing MerP\textsubscript{red} relative to the oxidized form. One of these is the unusually low \(pK_a\), 5.5, of the cysteine thiol at position 17. Its absence in C17S and C17A resulted in variants with stabilities similar to MerP\textsubscript{ox} rather than to MerP\textsubscript{red}. Interestingly, the Cys-17 thiol \(pK_a\) was also perturbed when Cys-17 was replaced by alanine or serine; this may be a result of structural changes occurring upon loss of the negative charge at position 17. Consistent with these notions, a variant in which Cys-17 was replaced with aspartate, preserving the negative charge, was almost as stable as the reduced form, and the Cys-14 thiol \(pK_a\) was not affected. Furthermore, the observation by NMR spectroscopy (36) that low pH alters the structure of MerP\textsubscript{red} but not MerP\textsubscript{ox} provides additional support for the proposal that a negative charge at position 17 helps to maintain the structure. The lowering to 5.5 of the \(pK_a\) of the Cys-17 thiol from a more typical 8.7 would be expected to stabilize MerP\textsubscript{red} by 4.3 kcal/mol, which is greater than the observed value of 2.7 kcal/mol. However, the conformational change in going from reduced to oxidized MerP involves movement of Cys-17 from a buried to exposed position and other associated structural changes that amount to more than simple removal of a thiolate (6, 36). Thus, the low \(pK_a\) of Cys-17 is not the sole determinant of the relative stabilities of MerP\textsubscript{ox} and MerP\textsubscript{red}.

Like MerP, proteins such as thioredoxin, protein-disulfide isomerase and DsbA contain a pair of redox-active cysteines separated by two amino acid residues. The ability of proteins to oxidize reduced glutathione, as reflected in the equilibrium stabilities of MerP\textsubscript{ox} and DsbA have hydrogen bonding of backbone amides to the sulfur of the low-\(pK_a\) thiol (43, 44). In reduced MerP, the amide of M12 appears to be close enough to hydrogen bond with, and stabilize the thiolate of MerP. In the set of 20 NMR structures of reduced MerP, the S-amide\textsubscript{Met12} and S-amide\textsubscript{H Met12} average distances are 3.24 and 2.61 Å, respectively, within the limits of 3.25–3.55 and 2.3–2.6 Å, respectively, expected for S-N H-bonds (45, 46). Unlike in DsbA, where His-32 appears to help stabilize the thiolate anion at C30 (38, 44), there are no charged residues in the vicinity of Cys-17 in MerP.

Another key feature of Cys-17 is its inaccessibility as measured by reactivity with iodoacetamide, a neutral solvent-borne thiol-reactive reagent. Thus, the intrinsic reactivity of Cys-17 thiolate with iodoacetamide is 1800 times lower than Cys-14 thiolate. This is consistent with the structure of MerP\textsubscript{red} which shows that Cys-14 is exposed on the surface while Cys-17 is buried (6). As discussed earlier, the low \(pK_a\) of the Cys-17 thiol appears to help stabilize MerP\textsubscript{red} relative to MerP\textsubscript{ox}, maintaining the protein in a form competent for Hg\textsuperscript{2+} binding. By keeping ionized Cys-17 away from the surface of the protein, undesirable reactions of this group, such as thiol exchange or oxidation, would also be minimized; surface-exposed Cys-14, which is not ionized at neutral pH, is not so susceptible to undesirable side reactions. Furthermore, by keeping the thiol groups well apart in the reduced protein, conversion to the oxidized form would be minimized.

A C-X-X-C motif with a low \(pK_a\) thiol has been well characterized in redox proteins such as thioredoxin and DsbA. In these proteins it is the exposed thiol in the cysteine redox pair that has a lowered \(pK_a\) (43, 44). This is undoubtedly related to the disulfide-exchange mechanism of these proteins, where a nucleophilic cysteine initiates attack on the polypeptide substrate (47). By contrast, the MerP C-X-X-C motif appears to be specialized for metal binding.

While the separation of thiol groups may be important in keeping MerP reduced and competent to bind Hg\textsuperscript{2+}, how can both thiols become coordinated to mercuric ion as has been indicated by NMR experiments (6)? A comparison of the structures of free and Hg\textsuperscript{2+}-liganded MerP indicates that Cys-17 migrates to the surface of the protein, in the process partially unravelling the amino terminus of the \(\alpha\)-helix of which it is a part (6); a similar structural change is observed after formation of MerP\textsubscript{ox} (36). A possible mechanism for this might be that Hg\textsuperscript{2+} initially binds to Cys-14 at the surface of the protein and then attracts the negatively charged thiol from its inaccessible position. The structural change that results may be important to allow loaded MerP to dock with one of the Hg\textsuperscript{2+}-transporting proteins (6, 7), but experimental evidence for docking is currently lacking.

The importance of Cys-17 thiol deprotonation for equilibrium binding of Hg\textsuperscript{2+} was investigated by examining mercuric ion binding at pH 4, where this residue is mostly un-ionized. These binding studies, carried out in the presence of competing cysteine ligand to minimize nonspecific binding, indicated little difference in binding parameters at pH 4 versus pH 7.3. This is not too surprising, since dissociation constants for Hg\textsuperscript{2+}(thiol)\textsubscript{2}
complexes are on the order 10^{-40} over a range of pH values (48). Therefore, it follows that the experimentally determined apparent K_a values are dominated by competitive Hg^{2+} binding to cysteine and MerP thiols, and would be unperturbed by the relatively insignificant contribution of the equilibrium between protonated and deprotonated thiols at pH 4 versus pH 7.3. In other words, extremely tight binding of Hg^{2+} to the deprotonated form would shift the equilibrium from the protonated form to the Hg complex. Indeed, NMR data for bidentate binding of Hg^{2+} to glutathione thiols showed that binding was tight over the pH range 1–13 (49). It therefore may be concluded that a fully deprotonated Cys-17 thiol is not essential for binding, are much more significant.

The low pK_a of Cys-17 may also play an important role in release of Hg^{2+} from MerP to the mercuric ion transport proteins, MerT and/or MerC. NMR studies have demonstrated that Hg^{2+} is rapidly exchanged among thiol ligands, such as those in Hg(glutathione)_2, via transient formation of an Hg(thiol)_3 complex (50). If transfer of Hg^{2+} from MerP to a thiol pair on MerT or MerC occurs via an Hg(thiol)_3 complex, the low pK_a of Cys-17 relative to the other two thiol pK_a values would favor it as a leaving group. However, it must be noted that despite its attractiveness as a hypothesis, evidence is currently lacking for direct transfer of Hg^{2+} between thiol pairs on different Mer proteins.

It is interesting to note that, although the structure of the metal-binding domain of the Menkes copper-transporting ATPase is very similar to that of MerP, no conformational change was observed upon metal binding to a Menkes domain (51). Furthermore, Cys-17 in the Menkes protein is exposed to solvent, and is thus unlikely to have a perturbed pK_a as in MerP. Use of the MTCXXC heavy metal binding motif to engineer metal binding sites into proteins thus may need to take into account the context of the cysteine residues to help control metal binding reactivity.

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