Communication

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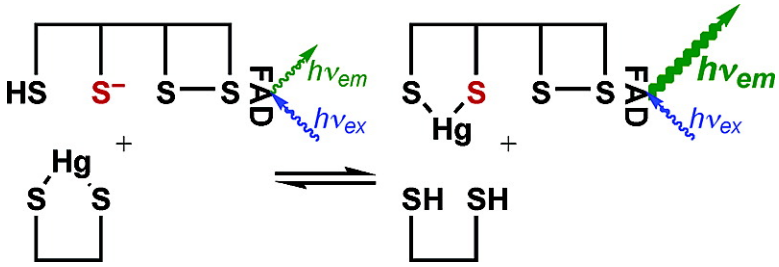

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Direct Monitoring of Metal Ion Transfer between Two Trafficking Proteins

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At elevated concentrations, both essential and nonessential metal ions pose acute challenges to biological systems due to their propensity to randomly coordinate and catalytically react with functional groups inside the cell.[1] Biological systems have responded with a number of metal ion trafficking proteins that specifically mobilize and/or eliminate metal ions.[2] Elucidation of the properties of proteins bound to their cognate metal ions is crucial to a better understanding of metal ion trafficking and potential pathways for toxicity in biological systems. While structures for several metal ion trafficking proteins have been reported,[3] analysis of the kinetics of metal ion transfer between proteins has lagged due to a lack of methods to monitor the rapid transfer. Taking advantage of the fluorescent properties of mercuric ion reductase (MerA), we report on the direct transfer of the metal ion Hg^{2+} between separately expressed N- and C-terminal domains of MerA.

MerA plays a pivotal role in bacterial mercuric ion detoxification by catalyzing the NADPH-dependent two-electron reduction of Hg^{2+} to Hg^0.[2c] It has two components, a multidomain catalytic core and an N-terminal domain (NmerA). The catalytic core is homologous with the flavin-disulfide oxidoreductase family that catalyzes reversible redox reactions with NADPH and disulfide/dithiol substrates.[4] NmerA shares sequence homology with a number of metal ion binding proteins, including metallochaperones, metal ion receptors, and soluble domains of metal ion transporters.[5] MerA contains three cysteine pairs, one in NmerA and two in the core (C-terminal and inner pairs), each of which contributes to the function of MerA in Hg^{2+} binding and reduction. The dithiol pair on NmerA has recently been shown to bind Hg^{2+} and transfer it to the C-terminal dithiol.[5] The latter pair is found on the flexible tail of the catalytic core and serves as a ligand exchange pathway to remove NmerA or other cellular thiols as Hg^{2+} travels from the exterior of the protein to the inner cysteine pair, where reduction to Hg^0 occurs.[2c,6] To monitor Hg^{2+} transfer between the N- and C-terminal cysteine pairs more easily, we cloned NmerA and the core separately.[5]

Studies of the pH dependence of enzyme-bound flavin fluorescence in wild-type catalytic core (CCCC) with the inner cysteines oxidized and the C-terminal cysteines reduced show two ionizations (pK_a's of 6.4 and 8.8) that both quench the fluorescence upon deprotonation (Figure 1). Mutational studies of the C-terminal cysteines (CCAC and CCCA) indicate the pK_a of 6.4 is attributable to the thiol of C-terminal Cys558, while the pK_a of 8.8 remains unchanged (Figure 1). Since the Cys558 thiolate is responsible for fluorescence quenching, we thought it may also serve as a sensitive probe for Hg^{2+} coordination; i.e., fluorescence would increase when Cys558 is bound to Hg^{2+}. To test this we examined the reaction of a Hg−NmerA complex with wild-type catalytic core with only the C-terminal cysteines reduced so that Hg^{2+} can transfer only between the N- and C-terminal cysteine pairs. As predicted, the fluorescence increased (Figure 2, ascending curve), demonstrating that Cys558 has become coordinated to Hg^{2+} and confirming that Hg−NmerA

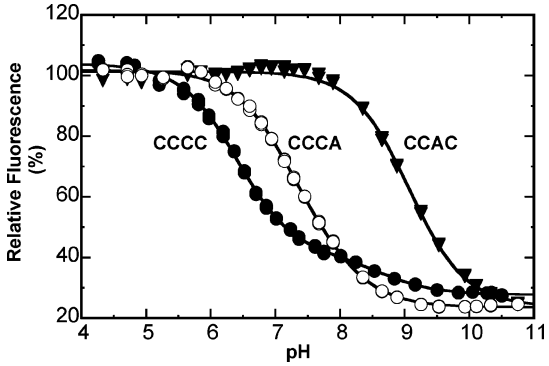


Figure 1. Fluorescence versus pH profiles of wild-type (CCCC) and mutant (CCAC and CCCA) catalytic core MerA's.

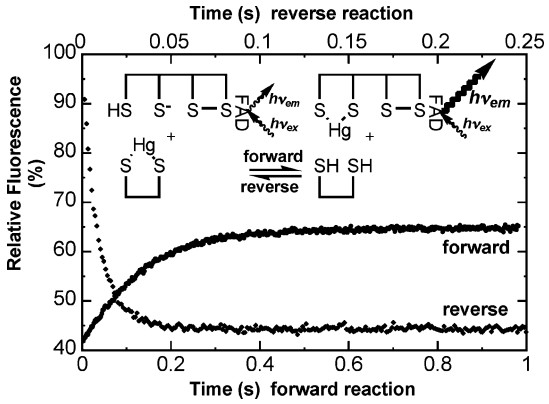


Figure 2. Transfer of Hg^{2+} between two proteins. Reaction conditions: 50 mM KPi, pH 7.3, 5 μM catalytic core (C-terminal reduced or Hg-bound), and 75 μM NmerA (Hg-bound or reduced).

delivers Hg^{2+} to the C-terminal cysteine pair, in agreement with our steady-state kinetic data.[5] In a control reaction of the core with $Hg(Cys)_2$, the final fluorescence equaled that of fully protonated enzyme, 100% (data not shown), suggesting that the reaction with Hg−NmerA reaches an equilibrium and therefore must be reversible. To test this, we synthesized Hg−CCCC with its inner cysteines oxidized and C-terminal cysteines bound to Hg^{2+} and looked for transfer of Hg^{2+} to reduced NmerA. The decrease in fluorescence (Figure 2, descending curve) demonstrates that the reaction is reversible.

The kinetic traces in Figure 2 (with NmerA:core = 15:1) reveal different rates and equilibrium end points for Hg^{2+} transfer in the two directions. Fits of the raw fluorescence data to a single exponential show the reverse reaction to be ~10-fold faster than the forward reaction. The magnitude of the fluorescence change indicates the final equilibrium distribution of Hg^{2+} between the two proteins at each NmerA concentration. With an initial fluorescence for CCCC of 42% compared with the fully bound control reaction (100%), the final fluorescence of ~62% in the

10.1021/ja052872c CCC: $30.25

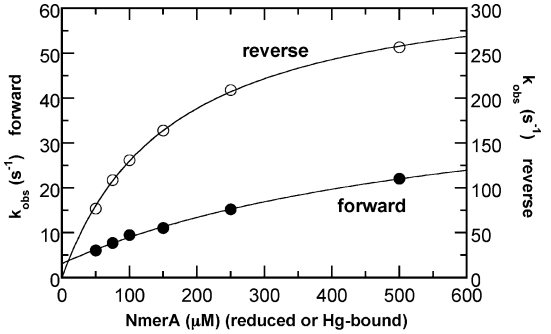
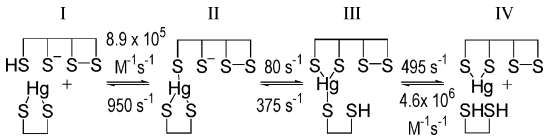


Figure 3. Plot of k_{obs} for Hg^{2+} transfer as a function of NmerA (reduced or Hg-bound) concentration in both directions.

Scheme 1



forward reaction of Figure 2 indicates ~35% of the core has become bound to Hg^{2+}. In the reverse reaction, the maximum fluorescence change demonstrates that 100% of Hg^{2+} transfers to NmerA under these conditions.

To tease out the kinetics and equilibria of Hg^{2+} transfer further, we examined the concentration dependence on the observed rate constant in both directions (Figure 3). In each case the data can be fit to a rectangular hyperbola but with a *y*-intercept of ~3 s^{-1} in the forward reaction and zero in the reverse (eq S1). Minimally, a hyperbolic concentration dependence for k_{obs} is indicative of a two-step process defined by k_{max} and $K_{1/2}$ values (Scheme S1) and an intermediate with the same properties as the starting species. Hyperbolic dependences in both directions requires at least two intermediates in the overall reaction, proposed here as three-coordinate Hg^{2+} complexes expected during ligand exchange[7] (Schemes 1 and S2). The intercept data give a measure of the extent of Hg^{2+} transfer in each direction. The finite *y*-intercept in the forward direction indicates an equilibrium is reached between quenched (I, II) and fully fluorescent (III, IV) species. The zero *y*-intercept in the reverse reaction indicates complete conversion to the quenched species (100% I, II) under those conditions. This analysis of the k_{obs} plots is entirely consistent with the raw data, where at every concentration tested the fluorescence change is <100% in the forward direction, but 100% in the reverse (Figures 4 and S1).

As described in the Supporting Information, raw fluorescence data from the forward and reverse reactions were converted into total concentrations of fluorescent (III + IV) and quenched (I + II) species and were fit simultaneously to the model shown in Scheme 1 (and Scheme S2) using the program Berkeley Madonna with initial estimates for the rate constants based on the k_{max} and $K_{1/2}$ values obtained from analysis of the plots in Figure 3. The fits to the forward reaction data are shown in Figure 4, and the values obtained from the simultaneous fit are shown in Scheme 1. Figure S1 shows fits to the reverse reaction. Potential errors in the values are discussed in the Supporting Information.

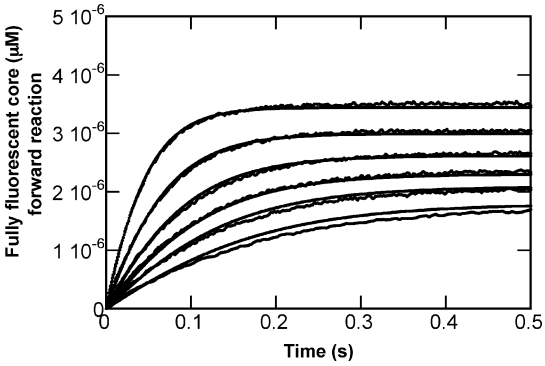


Figure 4. Model fits of Hg^{2+} transfer in forward direction using Berkeley Madonna with model shown in Scheme 1. Reaction of 5 μM catalytic core with increasing (50, 75, 100, 150, 250, and 500 μM) Hg−NmerA. (See Supporting Information for the reverse reaction fits.)

Surprisingly, under the conditions studied here the equilibrium for binding of Hg^{2+} to the two pairs of cysteines favors binding to NmerA. However, during normal turnover, the inner cysteines in the catalytic core are reduced and ready to accept Hg^{2+} from the C-terminal cysteine pair. We suspect that in the reduced state the inner cysteines provide a strong driving force to partition Hg^{2+} forward for reduction. Studies are underway to address this question.

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Supporting Information Available: Experimental procedures, further explanation of models with Schemes S1 and S2, eq S1, and Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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