Isolation of a High-Affinity Functional Protein Complex between OmcA and MtrC: Two Outer Membrane Decaheme c-Type Cytochromes of *Shewanella oneidensis* MR-1


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*Shewanella oneidensis* MR-1 is a facultatively anaerobic bacterium capable of using soluble and insoluble forms of manganese [Mn(III/IV)] and iron [Fe(III)] as terminal electron acceptors during anaerobic respiration. To assess the structural association of two outer membrane-associated c-type decaheme cytochromes (i.e., OmcA [SO1779] and MtrC [SO1778]) and their ability to reduce soluble Fe(III)-nitrilotriacetic acid (NTA), we expressed these proteins with a C-terminal tag in wild-type *S. oneidensis* and a mutant deficient in these genes (i.e., ΔomcA mtrC). Endogenous MtrC copurified with tagged OmcA in wild-type *Shewanella*, suggesting a direct association. To further evaluate their possible interaction, both proteins were purified to near homogeneity following the independent expression of OmcA and MtrC in the ΔomcA mtrC mutant. Each purified cytochrome was confirmed to contain 10 hemes and exhibited Fe(III)-NTA reductase activity. To measure binding, MtrC was labeled with the multiuse affinity probe 4,5′-bis(1,3,2-dithioarsolan-2-yl)fluorescein (1,2-ethanedithio), which specifically associates with a tetracysteine motif engineered at the C terminus of MtrC. Upon titration with OmcA, there was a marked increase in fluorescence polarization indicating the formation of a high-affinity protein complex (Kd < 500 nM) between MtrC and OmcA whose binding was sensitive to changes in ionic strength. Following association, the OmcA-MtrC complex was observed to have enhanced Fe(III)-NTA reductase specific activity relative to either protein alone, demonstrating that OmcA and MtrC can interact directly with each other to form a stable complex that is consistent with their role in the electron transport pathway of *S. oneidensis* MR-1.

As a facultatively anaerobic gram-negative bacterium, *Shewanella oneidensis* MR-1 possesses one of the more versatile respiration capabilities that have been demonstrated to date by a single microorganism. In addition to using O2 as a terminal electron acceptor during respiration, it uses a variety of alternative terminal electron acceptors, such as nitrate, fumarate, Fe(III) and Mn(III/IV) (4, 21, 23). To maintain its diverse respiration capabilities, *S. oneidensis* MR-1 has developed a sophisticated electron transport network that has been hypothesized to be responsible for its metabolic flexibility. This network is believed to be highly branched so that different terminal electron acceptors can be used under various growth conditions. Furthermore, to reduce insoluble metals such as Fe(III) and Mn(III/IV) at neutral pH in oxidizing environments, this network must be able to transfer electrons from the cytoplasmic membrane where electrons are generated to the extracellular surface of the outer membrane, where reduction is thought to occur (27). Identification of the proteins and their functions in mediating metal reduction is of considerable importance both from a standpoint of understanding bacterial physiology and in order to facilitate bioremediation of contaminated sites, since these metal-reducing activities may also function in the reduction and immobilization of toxic metals, including U(VI), Tc(VII), and Cr(VI) (25, 36–40).

The *c*-type cytochromes are the major components of the electron transport network of *S. oneidensis* MR-1, whose genome contains up to 42 open reading frames that encode putative *c*-type cytochromes. Most of them (33 out of 42) possess more than one heme-containing binding site. In addition, 5 out of 15 membrane-bound *c*-type cytochromes of *S. oneidensis* MR-1 are predicted to be localized to the outer membrane. This is in contrast to the membrane *c*-type cytochromes found in most other bacteria, which are commonly associated with the inner cytoplasmic membrane (12, 18). Because of their location, these outer membrane cytochromes are believed to be directly involved in reduction of Fe(III) or Mn(III/IV) oxides via either direct contact with these metal oxides or other components of the electron transport network (15). Indeed, two of these outer membrane *c*-type cytochromes, OmcA (SO1779) and MtrC (SO1778; also known as OmcB), were previously demonstrated to play an important role in the reduction of Fe(III) and Mn(III/IV) (4, 21, 23).

OmcA and MtrC are *c*-type cytochromes with 10 putative heme-binding sites and a lipoprotein consensus sequence (4, 22). Although omcA and mtrC are adjacent to each other on the genome of *S. oneidensis* MR-1, they are not in the same operon (23). Disruption of the genes encoding OmcA or MtrC has no effect on the ability of *S. oneidensis* MR-1 to reduce many electron acceptors, including nitrate, nitrite, or anthra-

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electron transport pathway of that OmcA and MtrC interact directly with each other to form tase specific activity than either protein alone, demonstrating this observation, the purified proteins are demonstrated to OmcA and MtrC copurify with one another. Consistent with acid (NTA). Upon expression in wild-type S. interac with other heme proteins or redox-active metabolites tion of Fe(III) or Mn(III/IV) oxide (4, 21, 23), it is unclear that MtrC plays a critical role in iron reduction through either citrate and Fe(III) oxide (4, 21). These latter results suggest that MtrC is involved in the proper localization of S. Oneidensis strain MR-1 containing plasmids pLS127 or pLS132 was cultured in 10 ml of LB plus 50 μg/ml kanamycin. After the culture was grown at 30°C until its optical density at 600 nm reached 0.6, t-arabinose was added to a final concentration of 1 mM. The cells were grown for another 17 h and harvested by centrifugation at 6,000 × g and 4°C for 15 min. The harvested cells were washed once with 30 ml of ice-cold buffer A (20 mM HEPES [pH 7], 150 mM NaCl) and stored at −20°C. To isolate recombinant OmcA, the cell pellets were resuspended with 30 ml of ice-cold buffer B (20 mM HEPES [pH 7], 5 mM β-mercaptoethanol), to which protease inhibitor (Roche Diagnostic, Indianapolis, IN) was added in accordance with the manufacturer’s instructions, and lysed by passage through a French press three times at 8,000 lb/in². The unbroken cells and debris were removed by centrifugation at 15,000 × g and 4°C for 30 min. The supernatant was transferred to a 60-ml ultracentrifugation tube and centrifuged at 150,000 × g for 1 h. The pellet (i.e., the membrane fraction that was dark red) was resuspended with 50 ml of ice-cold buffer C (buffer B plus 1% [wt/vol] n-octyl-β-D-glucopyranoside [OGP]), transferred to a 50-ml ultracentrifugation tube, and solubilized by rotating the tube at 4°C for 1 h. The unsolubilized proteins were removed by centrifugation at 15,000 × g and 4°C for 30 min. The supernatant was loaded onto a column (1 by 5 cm) of Ni²⁺-NTA agarose prequillified with buffer C. The column was washed with 25 ml of the following ice-cold buffers in sequential order: buffer C, buffer D (buffer C plus 150 mM NaCl) and 10% [vol/vol] glycerol), and buffer E (buffer D plus 40 mM imidazole). OmcA was eluted with 25 ml of buffer F (buffer E plus 500 mM NaCl), aliquoted, and stored at −20°C. Before measurement of Fe(III)-NTA reduction activity, aliquoted OmcA was thawed and changed to buffer G (20 mM HEPES [pH 7], 5 mM β-mercaptoethanol, protease inhibitor, 150 mM NaCl, 1% [wt/vol] OGP, 10% [vol/vol] glycerol) with a spin column of Microcon YM-10 (molecular mass cutoff of 10 kDa) from Millipore (Billerica, MA), and its protein concentration was determined. The procedures for purifying recombinant MtrC and OmcA by anion-exchange chromatography were identical. After membrane-bound MtrC or OmcA was solubilized as described above, the supernatant was loaded onto a 5-ml Econo-Pack High Q cartridge (Bio-Rad, Hercules, CA) that was equilibrated with buffer C. The cartridge was washed with 50 ml of the following ice-cold buffers in sequential order: buffer C, buffer D (buffer D plus 50 mM imidazole), aliquoted, and stored at −20°C. Before measurement of Fe(III)-NTA reduction activity, aliquoted MtrC was thawed and changed to buffer G (20 mM HEPES [pH 7], 5 mM β-mercaptoethanol, protease inhibitor, 150 mM NaCl, 1% [wt/vol] OGP, 10% [vol/vol] glycerol). After determination of their concentrations, the proteins were aliquoted and stored at −20°C. The yield was about 3 mg each for OmcA and MtrC. The identities of isolated heme-containing OmcA and MtrC were con- firmed by Western blot analysis with anti-V5 antibody (Invitrogen) and heme staining, and their purities were judged by staining with GelCode blue stain reagent after SDS-PAGE. Cloning, expression, and purification of MtrA (SO1777), a soluble decaheme c-type cytochrome of S. oneidensis MR-1, and determination of the absorption spectra and heme contents of purified OmcA and MtrC were carried out as described before (2, 32).

Membrane enrichment. To enrich the membrane of wild-type S. oneidensis MR-1, 100 ml of S. oneidensis MR-1 culture was grown and harvested in the same way as described in the protein purification section. After cells were resuspended in 30 ml of buffer B and lysed by passing the suspension through a French press three times at 8,000 lb/in², the cells lysates were centrifuged at 15,000 × g and 4°C for 30 min to remove unbroken cells. The supernatant was then further centrifuged at 150,000 × g and 4°C for 1 h. The pellets containing enriched membrane were resuspended with 100 ml of buffer C to solubilize proteins. After protein concentration determination, solubilized proteins were used for SDS-PAGE analysis.

Enrichment of the proteins copurified with MtrC. To identify the proteins copurified with MtrC, the MtrC isolated by anion-exchange chromatography was
loaded on a 10-cm-long tube containing 10% (wt/vol) acrylamide gel and further separated from other proteins by preparative SDS-PAGE with a Mini Prep Cell from Bio-Rad (Hercules, CA) according to the manufacturer’s protocol. A total of 10 mg of MtrC isolated by anion-exchange chromatography was used for several runs of preparative SDS-PAGE, and fractions (2 ml) were collected. The fractions without MtrC, which lacked visible color, were pooled, concentrated to about 1 ml by an Amicon Ultra Centrifugal Filter Device from Millipore, and used for mass spectrometry (MS) analysis.

Identification of the proteins copurified with MtrC by MS analysis. The enriched proteins were analyzed by two MS methods by following either a previously described protocol (8) or the instructions from the manufacturer. Briefly, for liquid chromatography in conjunction with Fourier transform ion cyclotron resonance (LC-FTICR) MS analysis, 50 μg of total protein was digested with trypsin (Promega, Madison, WI). Following clean-up on a strong cation-exchange SPE column (SUPELCO, Bellefonte, PA), the resulting peptides were identified with a 9.4 T FTICR mass spectrometer (Bruker Daltonics, Billerica, MA). The FTICR data were processed, and peptide sequences were determined following analysis in triplicate by the accurate mass and time tag approach developed at the Pacific Northwest National Laboratory (8, 28).

For matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) MS analysis, 10 μg of enriched proteins was separated by SDS-PAGE and the proteins were stained with GelCode blue. Individual bands of stained proteins were cut from the polyacrylamide gel. Each gel piece was then ground in a single 0.5-mL tube. The pieces were destained and dehydrated with 50% (vol/vol) acetonitrile/25 mM ammonium bicarbonate and 100% acetonitrile, respectively. After drying, each gel piece was covered with 15 μL of trypsin solution (20 ng/μL trypsin) and incubated at 37°C for 2 h. Peptides were then collected and resuspended in 10 μL of 25 mM ammonium bicarbonate in 50% (vol/vol) acetonitrile. Peptides were crystallized in α-cyano-4-hydroxycinnamic acid and analyzed with an Autospec II MALDI-TOF mass spectrometer (Bruker Daltonics) operated in reflector mode. The top 15 parent ions detected were selected for tandem MS, where 1,000 shots were collected for each corresponding fragment ion spectrum. A combination of peptide mass fingerprinting and peptide tandem MS data was submitted for identification with the Mascot tool. Multiple pieces of data were acquired, including combinations of peptide mass fingerprint and tandem MS data (Mowse scores with P < 0.05) or multiple tandem MS peptide matches.

Reductase activity of purified OmcA and MtrC. Transient kinetic measurements of absorbance changes at 552 nm were used to determine the oxidation rate of either OmcA or MtrC that were chemically reduced with sodium dithiothreitol by addition of electron acceptors, essentially as described previously (9, 16, 27, 41). Briefly, an SFA-20 stopped-flow apparatus (Hi-Tech Ltd., Salisbury, England) was used for mass spectrometry (MS) analysis.

The reaction solution, whose final volume was 210 μl in either 100 mM PIPES (pH 6.2 to 6.8) or HEPES (pH 6.8 to 8.2), contained 1.0 mM 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine monosodium salt (Ferrozine), 40 mM MgCl2, 0.2 mM NADH, 10 mM Fe(III)-NTA, 1% (wt/vol) OGP, 10% (vol/vol) glycerol, and 3 μg of purified MtrC or OmcA (or a combination of equal amounts of both proteins). In control solutions, either NADH, purified proteins, or Fe(III)-NTA was omitted. The reaction was prepared on ice, carried out at room temperature for 10 min, and terminated by placing the reaction plates on ice. The formation of an Fe(II)-3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine monosodium salt complex was measured spectrophotometrically at 562 nm essentially as described previously (6), with a SpectraMax UV/Vis spectrophotometer from Molecular Devices (Sunnyvale, CA). To determine whether NADH could reduce purified cytochromes, 1 mM NADH was added to an anaerobic glass cell (STARNA, Atascadero, CA) containing purified OmcA (80 μg), MtrC (80 μg), or MtrA (40 μg) in 1 ml of 100 mM HEPES (pH 7), 1% (wt/vol) OGP, and 10% (vol/vol) glycerol. The absorption spectrum of OmcA, MtrC, or MtrA was then recorded by a spectrophotometer (UV-2101 PC; Shimadzu, Japan). Controls included omission of NADH and replacement of NADH with 1 mM NADPH, 1 mM lactate, or H2. H2 was added by purging the solution with pure H2 for 30 min. All solutions were purged with 80% N2 and 20% CO2 (room temperature, 2 h) before use, and all reactions were performed in an anaerobic chamber (6, 10).

Labeling of MtrC with FlAsH-EDT2, and fluorescence polarization measurements. FlAsH-EDT2 was synthesized according to previously published protocols (1, 17). Before addition of 30 μM FlAsH-EDT2, 1 ml of MtrC (15 μM) in buffer P (50 mM HEPES [pH 7.5], 140 mM KCl, 5 mM β-mercaptoethanol, 0.1% [wt/vol] OGP) was incubated with 1.0 mM tris(carboxymethyl)-phosphate for 1 h at room temperature to reduce any disulfide bonds (5). The labeling reaction with FlAsH-EDT2 was carried out at 4°C overnight. After labeling, free FlAsH-EDT2 was removed by dialyzing the reaction solution in a dialysis cassette (molecular mass cutoff of 10 kDa; Pierce) against 1 liter of buffer P twice at 4°C overnight. The fluorescence emission spectra of FlAsH-bound to MtrC were recorded by a FluoroMax-2 fluorometer (SPEX, Edison, NJ) with excitation and emission slits of 5 nm and excitation at 500 nm. Fluorescent polarization (P) was measured as follows: \( P = \frac{I_{vH}}{I_{vH} + I_{vh}} \) (13).

Determination of binding affinity. Increases in the polarization of FlAsH-labeled MtrC were measured upon increasing amounts of OmcA, permitting detection of the formation of the MtrC-OmcA complex (i.e., [OmcA]bound) and calculation of the amount of unbound OmcA (i.e., [OmcA]free) as follows: \([\text{OmcA}]_{\text{bound}} = \left[ \frac{P - P_{\text{free}}}{P_{\text{max}} - P_{\text{free}}} \right] \times [\text{MtrC}]_{\text{total}} \) and \([\text{OmcA}]_{\text{free}} = [\text{OmcA}]_{\text{total}} - [\text{OmcA}]_{\text{bound}} \) (13).

The binding isotherm was fitted to the Hill equation as follows: \([\text{OmcA}]_{\text{bound}} = \left( \frac{[\text{OmcA}]_{\text{free}} ([K_a + ([\text{MtrC}]_{\text{total}})])}{1 + [\text{MtrC}]_{\text{total}}} \right) \times [\text{MtrC}]_{\text{total}} \). The slope of the Hill equation is the Hill coefficient associated with cooperative binding, and Min and Max are fitting parameters associated with the initial value and full range of the binding complex.

RESULTS

Expression and purification of OmcA and MtrC. Following induction, recombinant proteins OmcA and MtrC were expressed in the ΔomcA mtrC double mutant. After cell lysis and ultracentrifugation, OmcA and MtrC are principally found in the membrane fraction (data not shown). To facilitate purification, a screen with various detergents, including Triton X-100 and Tween-20, was implemented. Optimal solubilization of OmcA and MtrC from the membrane pellet was obtained with 1% (wt/vol) OGP (data not shown). Under these conditions, solubilized OmcA binds to Ni2+-NTA columns, permitting its purification by immobilized metal ion affinity chromatography (IMAC). Following elution, purified OmcA migrates as a single band on SDS-PAGE with an apparent mass of approximately 75 kDa (Fig. 1A). Similar results were obtained for OmcA expressed without a tetracysteine tag. The two purified recombinant OmcA proteins are nearly electrophoretically homogeneous and exhibit identical absorption spectra.

In contrast to OmcA, recombinant MtrC was refractory to IMAC purification following solubilization with variable amounts of OGP or other detergents. For this reason, anion-exchange chromatography was employed to purify MtrC solubilized in 1% (wt/vol) OGP. After exhaustive washing to release nonspecific binding proteins, MtrC was eluted in a buffer containing 150 mM NaCl and 1% (wt/vol) OGP. The eluted MtrC protein was close to electrophoretically homogeneous as judged by staining with GelCode blue (Fig. 1A), which revealed a predominant band of approximately 71 kDa. In all cases, the purified heme-containing proteins migrate as a single band on SDS-PAGE with electrophoretic mobilities similar to those previously reported for these heme-containing proteins (9, 27). Western immunoblot assays and heme staining confirm the identities of OmcA and MtrC (lanes 2 and 3, Fig. 1A). Contaminating proteins were identified by LC-FTICR and MALDI-TOF MS analyses following their separation.
No other protein is present in sufficient quantity to perturb the absorption spectra.

The absorption peaks of oxidized OmcA (Fig. 1B) and MtrC (Fig. 1C) were maximal at 408 nm (i.e., Soret \([\gamma]\) peak) (curve 1, Fig. 1B and C). When reduced with sodium dithionite, the Soret peak maximum shifted to 419 nm and characteristic \(\alpha\) Soret absorption peaks became prominent at 523 and 552 nm, respectively (curve 2, Fig. 1B and C). These redox-dependent changes in the absorption spectra of purified OmcA and MtrC are typical for \(c\)-type cytochromes and indicate that these purified proteins contain functional hemes. On the basis of the difference absorption coefficient of 11,300 M\(^{-1}\)cm\(^{-1}\) at 550 nm (9), the average heme contents of purified OmcA and MtrC were calculated to be 10.2 and 10.4, respectively, consistent with the prediction of 10 heme-binding sites identified in each polypeptide (4, 22).

**Functional activity of purified decaheme cytochromes.** The functional activities of the purified OmcA and MtrC proteins were measured, permitting an assessment of whether these membrane proteins are properly folded with all bound cofactors following their expression. After reduction by dithionite, OmcA or MtrC was rapidly mixed with Fe(III)-NTA and transient changes in optical density associated with the Soret absorption peak at 552 nm were measured (Fig. 2). There was a rapid decrease in absorbance consistent with the oxidation of OmcA or MtrC; fits to the transient decay indicate apparent rate constants of 1.5 ± 0.1 and 2.0 ± 0.2 s\(^{-1}\) for OmcA and MtrC, respectively. In contrast, no change in the absorption spectrum of either OmcA or MtrC was observed upon rapid mixing with another electron acceptor (e.g., NaNO\(_2\) or NaNO\(_3\)). These results indicate that the purified proteins are functional reductases with activity toward Fe(III)-NTA and are not readily oxidized by nitrate or nitrite, a finding that is consistent with prior in vivo measurements that mutation of either \(\text{omcA}\) or \(\text{mtrC}\) only affects the metal-reducing activity of \(\text{Shewanella}\) and does not affect its ability to reduce nitrate or nitrite (4, 22).

**NADH-dependent Fe(III)-NTA reductase activity.** The ability of NADH to reduce OmcA or MtrC was considered to explore alternative assays of measuring OmcA- or MtrC-mediated Fe(III)-NTA reduction, as NADH does not directly...
reduce this Fe(III) complex. Addition of NADH resulted in the reduction of MtrC or OmcA, as evidenced by characteristic changes in the absorption spectra associated with a shift of the Soret peak maximum to 419 nm and characteristic β and α Soret absorption peaks that became prominent at 523 and 552 nm, respectively. NADH-mediated reduction of OmcA and MtrC was specific, as neither glycerol nor an alternate electron donor such as NADPH, lactate, or hydrogen was able to reduce the oxidized proteins. Furthermore, NADH failed to reduce MtrA, a soluble decaheme c-type cytochrome (Fig. 3).

With NADH as the electron donor, the Fe(III)-NTA reductase activity of OmcA and MtrC was measured. Purified recombinant OmcA and MtrC displayed Fe(III)-NTA reductase activity, with similar specific activities of 1.5 and 1.6 μmol min⁻¹ mg⁻¹ (Fig. 4C), indicating that under these conditions each enzyme has an apparent turnover value of about 2 s⁻¹. Virtually identical turnover values were measured with NADH as an electron donor relative to that measured following mixing of the enzymes reduced by sodium dithionite with Fe(III)-NTA (Fig. 2), indicating that the NADH-dependent reduction of these enzymes is fast in comparison to the rate-limiting reduction of Fe(III)-NTA. Thus, the reduction of Fe(III)-NTA is directly mediated by OmcA and/or MtrC because (i) OmcA and MtrC are unable to reduce Fe(III)-NTA when NADH is omitted, (ii) NADH does not reduce Fe(III)-NTA when OmcA and MtrC are absent, (iii) neither OmcA nor MtrC reacts directly with 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine monosodium salt when Fe(III)-NTA is omitted, and (iv) purified MtrA exhibits no ferric reductase activity toward Fe(III)-NTA when NADH is used as an electron donor.

The optimal Fe(III)-NTA reductase activity of OmcA and/or MtrC occurred at temperatures between 20 and 37°C. Outside of this range, the activity was reduced significantly; no activity was found at 4°C, while about 25 to 60% of the original activity remained at 65°C (Fig. 4A). Both proteins were active over a pH range of 6.2 to 8.2. The maximal activity of OmcA was at pH 6.8, and that of MtrC was at pH 7.0 (Fig. 4B). OmcA purified from ΔomcA ΔmtrC mutant cells by anion-exchange chromatography also displayed Fe(III)-NTA reductase activity with a specific activity similar to that of OmcA purified by IMAC (data not shown), clearly demonstrating that Fe(III)-
NTA reductase activity is associated with the isolated OmcA polypeptide. When equimolar amounts of OmcA and MtrC were combined and assayed, the Fe(III)-NTA reductase specific activity increased by approximately 40% in comparison to that obtained by assaying either protein alone, demonstrating a synergistic reduction activity (Fig. 4C). The enhanced activity following the addition of equimolar amounts of OmcA and MtrC suggests a possible intermolecular interaction between OmcA and MtrC, which indicates that these proteins may function as a protein complex.

Copurification of OmcA and MtrC. To further assess possible interactions between OmcA and MtrC, OmcA was expressed and purified from wild-type S. oneidensis MR-1 cells. Following purification of tagged OmcA, two heme staining bands are apparent (lane 1 in Fig. 5). The upper band, with a molecular mass of approximately 75 kDa, is OmcA, as evidenced by immunoblot assays against the V5 tag (data not shown). The lower band, with an apparent mass of approximately 68 kDa, is MtrC, as indicated by Western immunoblot assays with an antibody against MtrC (lanes 4 and 6 in Fig. 5) (3). The mobilities of these proteins are, furthermore, similar to that of endogenous OmcA and MtrC present in enriched vesicles prepared from wild-type cells (lane 3 in Fig. 5); the broader band associated with OmcA following purification is consistent with the higher molecular mass of tagged OmcA, which contains a tetracysteine binding motif in addition to the V5 epitope and His6 tags. Additional confirmation of the identities of expressed OmcA is possible with a ΔomcA ΔmtrC double mutant, where it is apparent that following the purification of tagged OmcA, the lower band, with an apparent molecular mass of approximately 68 kDa, no longer copurified with OmcA (lane 2 in Fig. 5). Under these conditions, there is also a loss of density near the bottom of the 75-kDa band, consistent with the disruption of omcA. These results indicate that OmcA and MtrC associate and form part of a functional Fe(III)-NTA reductase complex. The ability of the tagged OmcA protein to copurify with endogenous OmcA suggests that this is a multimeric complex involving both tagged and endogenous OmcA in association with MtrC.

Direct binding between OmcA and MtrC. The failure of recombinant MtrC to bind to Ni²⁺-NTA agarose prevented direct determination of whether OmcA could be copurified with recombinant MtrC under identical conditions. To validate the association between MtrC and OmcA, we selectively labeled the tetracysteine tag encoded on purified MtrC with...
FlAsH-EDT₂ (Fig. 6A, insert), permitting the use of fluorescence spectroscopy to monitor binding through changes in polarization. This assay takes advantage of the rigid association between the tetracoordinate FlAsH labels and protein molecules, which prevents free rotation of the fluorophore (5, 17). Thus, changes in the overall rotational dynamics of MtrC can be measured by fluorescence polarization, which is sensitive to the overall size of the protein complex.

In the absence of OmcA, the steady-state polarization of FlAsH-labeled MtrC is approximately 0.175. Upon addition of OmcA, there is a progressive increase in the steady-state polarization, with a half point at an [OmcA]/[FlAsH-MtrC] ratio of close to 2, that approaches a limiting value of approximately 0.23 (Fig. 6A). In contrast, addition of purified MtrA, which is also implicated in the Fe(III) electron transport pathway (3, 27, 32), did not affect the measured polarization values for FlAsH-labeled MtrC (Fig. 7), emphasizing the specificity of the interaction. Furthermore, binding was reduced by addition of salt, resulting in a shift in the OmcA-dependent increase in polarization to higher ratios of OmcA relative to MtrC (Fig. 7), emphasizing the specificity of the association between monomeric OmcA and MtrC.

**Determination of binding affinities.** Binding affinities were calculated for the formation of the MtrC-OmcA complex with the Hill equation (the last equation in Materials and Methods). All binding isotherms were simultaneously fitted by assuming a common mechanism (i.e., linkage between the Hill coefficients) in which binding affinities were dependent upon the salt concentration (Fig. 6B). Calculated fits indicate that the apparent dissociation constant (Kₐ) varies between 0.5 and 1.0 μM, depending upon the salt concentration (Fig. 6C). Given that both MtrC and OmcA are membrane proteins that are constrained in a two-dimensional lattice, these results suggest the formation of a stable oligomeric complex. Furthermore, the Hill coefficient (n) was 1.6 ± 0.2, indicating positive cooperativity of binding. These latter results suggest that two OmcA molecules associate with each MtrC molecule, consistent with the results of the pull-down affinity purification measurements where the intensity of heme staining of both endogenous and recombinant OmcA bands is approximately twice that of MtrC (lane 1 in Fig. 5). These results strongly suggest that more than one OmcA may associate in complex with MtrC.

To further investigate the possible association between multiple OmcA protein molecules with each MtrC molecule to form an oligomeric protein complex, we took advantage of the fact that OmcA with a tetracysteine tag engineered at the C-terminus forms a stable dimer under nonreducing conditions (i.e., Kₐ = 0.4 ± 0.1 μM and n = 1.7 ± 0.2). Upon dimerization, Kₐ = 0.4 ± 0.1 μM and n = 1.0 ± 0.1. In all cases, experimental conditions involved 0.5 μM FlAsH-labeled MtrC in 50 mM HEPES (pH 7.5), 140 mM KCl, and 2% OGP.
DISCUSSION

We have cloned, expressed, and purified the outer membrane-associated decaheme cytochromes OmcA and MtrC to electrophoretic homogeneity, permitting, for the first time, direct measurements of their metal reductase activity and functional association. Both purified cytochromes are functional Fe(III)-NTA reductases that lack activity toward nitrite or nitrate. Independently of whether dithionite or NADH is used to reduce OmcA or MtrC, virtually identical rates of reduction of Fe(III)-NTA are observed (2 s\(^{-1}\)) (Fig. 2 and 4). Thus, the reduction of OmcA or MtrC by NADH is fast in comparison to the reduction of Fe(III)-NTA, which is rate limiting (2 s\(^{-1}\)). OmcA cooperatively associates with MtrC (K\(_d\) < 500 nM) and forms an oligomeric protein complex involving multiple OmcA subunits with enhanced Fe(III)-NTA reductase activity. The high-affinity association between OmcA and MtrC is consistent with the copurification of these proteins. In contrast, another decaheme cytochrome (i.e., MtrA) does not bind with this complex, consistent with the absence of other heme-containing proteins following its purification (32). These results suggest that prior observations of MtrA in promoting electron transfer between the inner and outer membranes of Shewanella oneidensis MR-1 would involve transient low-affinity associations (27).

Prior measurements have identified the presence of OmcA, MtrC, and other c-type cytochromes in the outer membranes of Shewanella that function to couple conserved electron transfer chains present in the inner membrane to mediate the reduction of insoluble metals (20, 23). The involvement of the outer membrane decaheme cytochrome MtrC in facilitating the reduction of iron oxides in Shewanella was first identified following gene disruption, although residual metal-reducing activity remains (4). Likewise, OmcA has been reported to participate in the reduction of metal oxides [i.e., Mn(IV) and Fe(III)], although it does not appear to be required for the reduction of chelated soluble iron (9, 23). Our measurements, demonstrating a functional interaction between purified OmcA and MtrC, which facilitates the Fe(III)-NTA reductase activity of these enzymes, is consistent with the fact that neither OmcA nor MtrC is absolutely required for growth, as both enzymes have some capacity to reduce Fe(III)-NTA. Since OmcA and MtrC reduce Fe(III)-NTA independently, this functional redundancy may be the reason that the ΔomcA mutant is still capable of reducing soluble iron(III) complexes (9, 23). An OmcA homolog from Shewanella frigidimarina that is 55% identical to OmcA of S. oneidensis MR-1 was previously purified free from other heme-containing proteins, and this preparation was reported to reduce soluble Fe(III) complexes. Further, OmcA of S. frigidimarina was found to contain 10 hemes, consistent with sequence-based predictions (9). Our present results are consistent with these prior measurements and sequence-based predictions that MtrC contains 10 hemes.

The reduction of OmcA and MtrC by NADH is specific, as other electron donors, such as NADPH, lactate, and H\(_2\), failed to reduce OmcA or MtrC. In addition, NADH does not reduce MtrA, and thus MtrA is not capable of any NADH-dependent Fe(III)-NTA reductase activity. In contrast, following reduction by sodium dithionite, MtrA is known to reduce soluble Fe(III)-EDTA as well as Fe(III)-maltol (27). Thus, differences in assay conditions underline differences in the observed ability of MtrA to reduce soluble Fe(III) complexes. NADH dehydrogenase is not involved in the observed NADH-mediated reduction of either OmcA or MtrC, since both enzymes are reduced by NADH following their purification to near electrophoretic homogeneity. Furthermore, contaminating proteins (<1% of the total protein) were identified by MS analysis and none of the subunits associated with predicted NADH dehydrogenase from MR-1 were found to copurify with MtrC by two different MS analyses. The failure to identify the subunits of NADH dehydrogenase from our sample is not caused by the limitation of MS analyses as some subunits of NADH dehydrogenase were previously identified from the membrane fraction used in our purification (J. K. Fredrickson, unpublished data).

The ability of NADH to directly reduce either OmcA or MtrC is puzzling, as physiological concentrations of NADH are not expected to be present in the periplasm. Furthermore, the polypeptide sequence of neither OmcA nor MtrC is predicted to contain a Rossmann dinucleotide binding fold (commonly associated with NADH binding) (30). Thus, the mechanism and physiological significance associated with the ability of NADH to reduce either OmcA or MtrC remain unclear. Nevertheless, the ability of NADH to reduce periplasmic c-type cytochromes purified from Geobacter sulfurreducens has been previously observed (31). While the physiological significance of the NADH-dependent reduction of periplasmic and outer membrane-associated proteins remains unclear, from a practical point of view the specificity of NADH in the reduction of either OmcA or MtrC has important experimental advantages in the measurement of their kinetic properties. This latter advantage is highlighted by the fact that similar turnover values (i.e., 2 s\(^{-1}\)) are observed irrespective of whether NADH is coupled to enzyme turnover or rapid mixing is used to measure the transient oxidation of the dithionite-reduced enzyme by Fe(III)-NTA (Fig. 2 and 4). These latter results indicate that the association and reduction of the highly soluble Fe(III)-NTA complex (where diffusional barriers would be expected to be minimal) represent the rate-limiting step of the reaction (Fig. 9). Taken together, all of these results clearly demon-
strate that both purified OmcA and MtrC have Fe(III)-NTA reductase activity and ensure that the reported measurements of binding between purified OmcA and MtrC are physiologically significant.

The Fe(III)-NTA-reducing activities of OmcA and MtrC are 1.5 and 1.6 μmol min⁻¹ mg⁻¹, respectively, which are the highest Fe(III)-NTA reductase activities reported to date (10, 16). Synergistic reduction activity of OmcA and MtrC toward Fe(III)-NTA further supports the direct interaction between OmcA and MtrC. The measured affinity suggests that these proteins exist as a stable complex, and their affinity is comparable to that of other proteins that form stable complexes, including the soluble proteins ferredoxin I and pyruvate-ferredoxin oxidoreductase from Desulfovibrio africanus (Kd = 38.5 μM), soluble cytochrome c and membrane-bound complex III of beef mitochondria (Kd = 0.2 μM), subunits of the photosynthetic reaction center of Rhodobacter sphaeroides (Kd = 4 μM), and the integral membrane proteins phospholamban and Ca²⁺-ATPase in cardiac muscle (Kd = 140 μM), in which approximately 70% of the Ca-ATPase is in complex with phospholamban (14, 26, 33, 34). In the case of membrane proteins, the stability of the complex is considerably enhanced by the diffusional constraints associated with the membrane environment. In addition, polarization measurements of binding between purified proteins indicate that OmcA and MtrC form a heterotrich in which two OmcA protein molecules interact with one MtrC protein molecule. Consistent with these binding measurements, endogenous OmcA was copurified with recombinant and tagged OmcA along with endogenous MtrC at an apparent ratio of approximately two OmcA protein molecules per MtrC molecule. Lane 3 in Fig. 5 represents the relative abundance of OmcA and MtrC in wild-type cells, which is unrelated to the stoichiometry of the isolated complex (Fig. 5).

The formation of a stable complex between MtrC and OmcA is consistent with prior dynamic models in which dehydrogenases in the inner membrane couple the oxidation of NADH and a range of different substrates through classical oxidative pathways involving quinol intermediates that function to reduce the inner membrane protein CymA that are then coupled to the outer membrane through soluble periplasmic proteins (e.g., MtrA and Cct) that function as intermediate electron carriers (4, 19, 24, 27, 29). These results are, furthermore, consistent with the ability of MtrA to couple electron transport between spatially distant reductases following its expression in Escherichia coli (27). In this model, the integral membrane protein MtrB, previously demonstrated to play a critical role in Fe(III)-NTA reduction, is suggested to represent a protein scaffold that permits the transient association between reduced periplasmic proteins and the OmcA-MtrC protein complex. Thus, depending on the metabolic needs of the organism, different redox partners can couple with either OmcA or MtrC as a means to maintain critical metabolic pathways important to the energy metabolism of the organism.

The ability to couple a range of different metabolic pathways to mediate metal reduction through a versatile electron transport chain involving OmcA and MtrC underlies the ability of Shewanella to colonize and populate anaerobic environments and represents an important understanding of how metabolic reductase activities can function in the reductive immobilization of toxic metals in contaminated sites. Future experiments should measure the possible association and function of a reconstituted OmcA-MtrC complex against solid metals to determine whether binding and metal reduction involve a direct association with individual proteins.

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