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An octaheme *c*-type cytochrome from *Shewanella oneidensis* can reduce nitrite and hydroxylamine

Sally J. Atkinson[a], Christopher G. Mowat[a], Graeme A. Reid[b], Stephen K. Chapman[a,*]

[a] *EaStCHEM, School of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, UK*
[b] *Institute of Structural and Molecular Biology, University of Edinburgh, Mayfield Road, Edinburgh EH9 3JR, UK*

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Abstract A *c*-type cytochrome from *Shewanella oneidensis* MR-1, containing eight hemes, has been previously designated as an octaheme tetrathionate reductase (OTR). The structure of OTR revealed that the active site contains an unusual lysine-ligated heme, despite the presence of a CXXCH motif in the sequence that would predict histidine ligation. This lysine ligation has been previously observed only in the pentaheme nitrite reductases, suggesting that OTR may have a possible role in nitrite reduction. We have now shown that OTR is an efficient nitrite and hydroxylamine reductase and that ammonium ion is the product. These results indicate that OTR may have a role in the biological nitrogen cycle.

Keywords: Octaheme tetrathionate reductase; Cytochrome *c*; Nitrite; Hydroxylamine; Nitrogen cycle

1. Introduction

Shewanella oneidensis MR-1 is a Gram-negative γ-proteobacterium that was initially isolated from Lake Oneida, NY. Genomic analysis of this metal-reducing bacterium has identified 42 genes encoding *c*-type cytochromes (recognised using the *c*-type heme attachment motif, CXXCH), a large proportion of which contain multiple heme groups [1]. This profusion of cytochromes is thought to confer a wide respiratory flexibility upon the organism by allowing it to utilise a diverse range of electron acceptors. These include: nitrite, nitrate, thiosulfate, sulfate, fumarate, and metal ions such as Fe(III) and Mn(IV).

A putative octaheme cytochrome (the product of gene SO4144 [2]), containing eight CXXCH motifs and located in the periplasm, has been identified. This protein was found to have a novel sequence, with no homologs of known function, and has been overexpressed in *Shewanella frigidimarina* EG301 [3]. The protein has been purified and found to have a molecular weight of 54.5 kDa, and its ability to reduce tetrathionate led to its assignment as an octaheme tetrathionate reductase (OTR). The crystal structure of this protein has been solved to 2.2 Å resolution, revealing a novel fold [3]. Despite the presence of eight conventional (CXXCH) heme-attachment motifs, only seven of the hemes are bis-histidine-ligated with one unusual lysine-ligated heme (heme 2) at the active site. This unconventional lysine heme ligation has been previously observed only for the heme 1 binding site of the pentaheme cytochrome *c* nitrite reductases from *Wolinella succinogenes* [4] and other bacteria, but in these cases the lysine ligand is located in a CXXCK motif.

There is considerable similarity between the arrangement of the hemes in the structure of OTR with those found in other multiheme cytochromes. These include the octaheme hydroxylamine oxidoreductase from *Nitrosomonas europaea* and the pentaheme nitrite reductases from *W. succinogenes*, *Escherichia coli* and *Sulfurospirillum deleyianum* [5]. These enzymes are involved in the interconversion of nitrogen-containing species in the biological nitrogen cycle, which has led to the suggestion that OTR may have a similar role.

In this paper, we introduce evidence to support this suggestion, showing that OTR is catalytically active with a range of nitrogenous compounds.

2. Materials and methods

2.1. Protein production and purification

OTR was produced in the *Shewanella frigidimarina* strain EG301 [6]. Cultures were grown in LB broth supplemented with streptomycin and kanamycin and induced using isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation and pellets were resuspended in Buffer A (10 mM Tris–HCl, pH 8.4). Cell lysis was initiated by incubation with egg white lysozyme (Sigma) for 30 min and completed by ultrasonication on ice. Cell debris was removed by centrifugation and the cell-free extract retained for column chromatography.

The cell-free extract was applied to a DE-52 column (Whatman), washed with Buffer A and eluted with 0.1 M NaCl. The protein fraction was further purified using a Q-Sepharose column and FPLC system (GE Healthcare), using a linear NaCl gradient (0–500 mM NaCl) in Buffer A. OTR was eluted at 100 mM NaCl. The OTR-containing fraction was then applied to two successive hydroxyapatite columns (BioRad Chemicals) and eluted firstly using a single 100 mM K_2HPO_4 step and secondly over 50 mM and 100 mM steps. Protein purification was monitored using SDS–PAGE.

2.2. Steady-state kinetic analysis

Steady-state kinetic analysis of substrate reduction was followed at 25 °C using an adaptation of the technique described by Turner et al. [7]. The substrate-dependent reoxidation of reduced methyl viologen was monitored at 600 nm using a Shimadzu UV-PC 1501 spectrophotometer. To ensure anaerobicity, the spectrophotometer was housed in a Belle Technology glovebox under a nitrogen atmosphere ($[O_2]$ <5 ppm).

*Corresponding author. Fax: +44 131 650 6453.
E-mail addresses: S.K.Chapman@ed.ac.uk, skc01@staffmail.ed.ac.uk (S.K. Chapman).

Abbreviations: OTR, octaheme tetrathionate reductase; IPTG, isopropyl-β-D-thiogalactopyranoside

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Bulk reduction of methyl viologen was carried out in anaerobic conditions at 25 °C as a 5 mM solution in 1.5 M NaCl. The electrodes used were platinum with an Ag/AgCl reference electrode. The potential of the solution was held at −550 mV vs. SHE using an Autolab PGSTAT10 potentiostat until reaction completion was indicated by cessation of current flow.

The assay buffer contained 50 mM Tris–HCl, pH 7.0. Reduced methyl viologen was added to the assay buffer so that a reading of ~1 absorbance unit was obtained (corresponding to ~80 μM). A known concentration of enzyme was added and the reaction initiated by addition of substrate at a range of concentrations (0–500 mM).

Steady-state thiosulfate oxidation was assayed under the same buffer conditions using ferricyanide as the electron acceptor. Thiosulfate oxidation is observed as a decrease in absorbance at 420 nm as ferricyanide (ε_{420} = 1.01 mM^{-1} cm^{-1}) is reduced by the enzyme.

Kinetic parameters K_M and k_{cat} were determined from steady-state results over a range of substrate concentrations fitted to the Michaelis–Menten equation by a least squares regression analysis (Microcal Origin software).

2.3. Ammonium detection

The substrate reduction assay mixtures were analysed for ammonium ions using a modified version of the indophenol method [8].

3. Results and discussion

The ability of OTR to catalyse the reduction of a number of substrates was assayed. Reductase activity was demonstrated with nitrite and hydroxylamine in addition to tetrathionate (Table 1), but no activity was observed using other sulfur-containing species (SO_3^{2-}, SO_4^{2-}). Thiosulfate oxidation was also detected. The product of the reduction of nitrite and hydroxylamine by OTR, directly detected in solution, was conclusively shown to be ammonium ion. Such activity is consistent with an enzyme that plays a role in the biological nitrogen cycle, with the conversion of nitrite to ammonium representing a short circuit in this cycle.

The biological nitrogen cycle is characterised by a number of metalloenzymes that catalyse redox reactions interconverting nitrogen species across the range of their oxidation states from nitrate (+5) to ammonia (−3), via a range of intermediates including dinitrogen, nitrite, nitric oxide, nitrous oxide and hydroxylamine. The structures of several of these metalloenzymes have been solved and, in this instance, those of the pentaheme cytochrome *c* nitrite reductases (NrfA) [4,9,10] and hydroxylamine oxidoreductase (HAO) [11] are of particular interest.

Analysis of the structures of these enzymes shows significant similarities in their arrangements of heme groups and this conservation of heme architecture is also found in OTR (Fig. 1). Seven of the eight hemes of OTR and HAO are superposable. The active sites of these enzymes are contained in identical three-heme clusters, with the actual active site hemes found flipped to either side of a seven heme chain in each case. This three-heme active site module is also found in the NrfA structures; in fact, hemes I–V of NrfA are almost exactly superposable with hemes II–VI of OTR, with this fit even extending to the substrate binding location and the position of the lysine ligating the active site heme (Fig. 2). These similarities are perhaps even more notable when it is considered that there is no sequence or structural similarity between these three enzymes. This structural conservation has led us to speculate that OTR may also be involved in the reactions of the nitrogen cycle and this hypothesis is supported by the ability of the enzyme to reduce nitrite and hydroxylamine, as we have shown above.

Table 1
Steady state kinetic data for the reactions of OTR with various substrates at 25 °C, pH 7.0

Substrate	Product	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})
Nitrite, NO_2^-	Ammonium ion, NH_4^+	2.8 ± 0.4	0.0052 ± 0.0010	5.3×10^5
Hydroxylamine, NH_2OH	Ammonium ion, NH_4^+	849 ± 7	2.2 ± 0.4	3.9×10^5
Tetrathionate, $S_4O_6^{2-}$	Thiosulfate, $S_2O_3^{2-}$	19.9 ± 0.6	0.07 ± 0.01	2.9×10^5
Thiosulfate, $S_2O_3^{2-}$	Tetrathionate, $S_4O_6^{2-}$	16.8 ± 0.9	7.1 ± 2.5	2.4×10^3

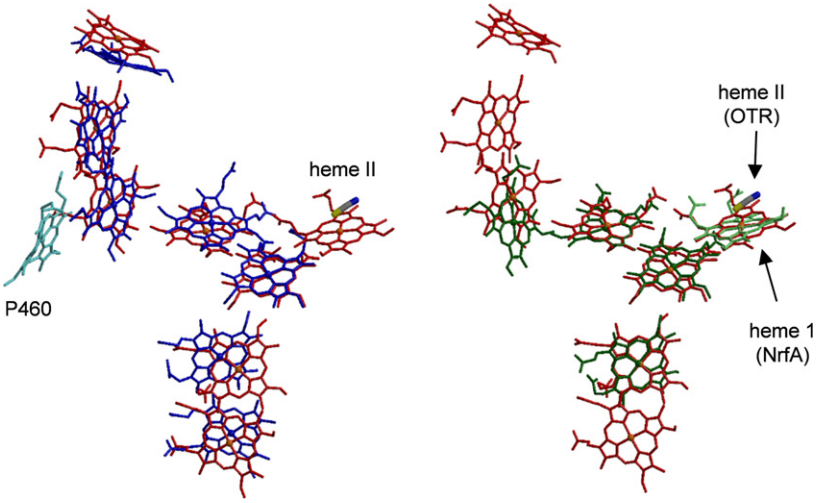

Fig. 1. Comparison of the heme arrangement of OTR (in red) with that in HAO (left, blue) and in NrfA (right, green).The active site P460 heme in HAO is shown in light blue and it can be seen that only the active site hemes are excluded from the superposition.