

Mutations in FMN binding pocket diminish chromate reduction rates for Gh-ChrR isolated from *Gluconacetobacter hansenii*

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Received 16 April 2013; revised 18 May 2013; accepted 26 May 2013

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ABSTRACT

A putative chromate ion binding site was identified proximal to a rigidly bound FMN from electron densities in the crystal structure of the quinone reductase from *Gluconacetobacter hansenii* (Gh-ChrR) (3s2y.pdb). To clarify the location of the chromate binding site, and to understand the role of FMN in the NADPH-dependent reduction of chromate, we have expressed and purified four mutant enzymes involving the site-specific substitution of individual side chains within the FMN binding pocket that form non-covalent bonds with the ribityl phosphate (*i.e.*, S15A and R17A in loop 1 between β 1 sheet and α 1 helix) or the isoalloxazine ring (E83A or Y84A in loop 4 between the β 3 sheet and α 4 helix). Mutations that selectively disrupt hydrogen bonds between either the N3 nitrogen on the isoalloxazine ring (*i.e.*, E83) or the ribitylphosphate (*i.e.*, S15) respectively result in 50% or 70% reductions in catalytic rates of chromate reduction. In comparison, mutations that disrupt π - π ring stacking interactions with the isoalloxazine ring (*i.e.*, Y84) or a salt bridge with the ribityl phosphate result in 87% and 97% inhibition. In all cases there are minimal alterations in chromate binding affinities. Collectively, these results support the hypothesis that chromate binds proximal to FMN, and implicate a structural role for FMN positioning for optimal chromate reduction rates. As side chains proximal to the β 3/ α 4 FMN binding loop 4 contribute to both

NADH and metal ion binding, we propose a model in which structural changes around the FMN binding pocket couples to both chromate and NADH binding sites.

Keywords: Bioremediation; Chromate Reduction; Enzyme Redesign; Site-Specific Mutagenesis; Chromate Reductase; Flavin Mononucleotide; Promiscuous Enzyme Activities

1. INTRODUCTION

Chromate [Cr(VI)] is a toxic pollutant that commonly leaches into ground water [1]. Recently it has been found that some bacteria express enzymes that are able to bind and reduce toxic Cr(VI) to form nontoxic Cr(III) as an intracellular precipitate, offering a cost-effective strategy of bioremediation [2,3]. Two enzyme classes are known to reduce chromate [3]. One class involves a metal reductase enzyme in the outer membrane that couples through a complex series of specialized electron transfer proteins [4-7]; the second class involves a cytosolic NAD(P)H-dependent chromate reductase (*i.e.*, ChrR) that functions autonomously [2,8,9]. A major advantage of the latter class of enzymes is their ability to concentrate and sequester reduced chromate [*i.e.*, Cr(III)] within the reducing environment of cells [10-12].

ChrR acts on quinones and other organic substrates (e.g., cancer prodrugs) through a ping-pong bi-bi (double displacement) mechanism whereby NAD(P)H and organic substrates bind sequentially, such that NAD(P)H reduces FMN prior to substrate binding and reduction [3,12-14]. In contrast, the reduction of chromate involves

an ordered bireactant mechanism, whereby chromate must first bind prior to the association of NAD(P)H [8]. Following electron transfer and reduction of chromate, NAD(P)⁺ must dissociate prior to the release of the reduced chromate. Consistent with this latter mechanism, the crystal structure of ChrR (3s2y.pdb) suggests a likely ion binding site for chromate proximal to FMN within the NAD(P)H binding pocket [8]. As chromate reduction by ChrR involves an adventitious enzyme activity [9] with a low catalytic rate and binding affinity ($k_{\text{cat}} = 0.25 \text{ sec}^{-1}$; $K_m = 0.24 \text{ mM}$) [8,13], the precise role of the FMN cofactor in chromate reduction remains uncertain. To test the prediction that chromate binds proximal to FMN, and to better understand the possible role of FMN in promoting the binding and reduction of chromate, we have used site-directed mutagenesis to assess how modifications in the FMN binding pocket affect chromate binding and its NADH-dependent reduction. Consistent with the proposal that FMN plays a catalytic role in chromate reduction, we observe substantial decreases in catalytic rates of chromate reduction upon the site-directed mutagenesis of four different FMN ligands (S15A, R17A, E83A, and Y84A) in the FMN binding pocket.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

All chemicals were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO) unless indicated otherwise. Ni-NTA resin was purchased from Qiagen Inc. (Valencia, CA). Isopropyl-D-1-thiogalactopyranoside (IPTG), agar, and LB medium were purchased from Fisher Scientific (Wilmington, MA). All plasmid purification kits were from Qiagen Inc. (Valencia, CA).

2.2. Site-Directed Mutagenesis

The DNA sequence of the Gh-ChrR gene from *Gluconacetobacter hansenii* ATCC 23769 (ZP_06834583), plus four single-codon mutants (S15A, R17A, E83A, Y84A and Y129N) were synthesized, and inserted into the expression vector pJexpress411 (DNA 2.0 Inc., Menlo Park, CA, USA) such that a 6-histidine tag was present at the C-terminus of the gene product. After that, mutant-expressing plasmids were codon optimized for expression in *E. coli*. All desired mutations were verified by DNA sequencing.

2.3. Protein Expression and Purification

Plasmids containing the gene encoding Gh-ChrR were transformed into *E. coli* BL21 (DE3) and recombinant protein expression was induced by the addition of IPTG to the medium (0.02 mM final concentration). Transformed BL21 cells in ice cold lysis buffer [0.3 M NaCl,

50 mM K_2HPO_4^- (pH 8.0)] were broken by repeated sonication (1 minute \times 3) (Branson Ultrasonic, Danbury, CT). Supernatants were collected following centrifugation and protein was affinity purified using a Ni-NTA resin, essentially as previously described [8].

2.4. Chromate Reduction Activity Assays

Reduction activity of chromate by Gh-ChrR and its mutants were measured at 37°C in 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.1 mM NADH, and 5 μM Gh-ChrR as a function of chromate concentration, where rates of NADH consumption were measured using absorbance changes at 340 nm, essentially as previously described [8]. Kinetic constants (K_m and V_{max}) were fit to a Michaelis-Menten equation. All measurements were conducted in triplicate.

3. RESULTS AND DISCUSSION

3.1. Inhibition of Chromate Reduction by Mutation of FMN Ligands

To investigate the role of FMN in the NADPH-dependent reduction of chromate, we have expressed Gh-ChrR in *E. coli* and purified mutant enzymes involving site-specific substitution of side chains that respectively position FMN either through the ribityl phosphate (*i.e.*, S15A and R17A in loop 1 between $\beta 1$ sheet and $\alpha 1$ helix) or the isoalloxazine ring (E83A or Y84A in loop 4 between the $\beta 3$ sheet and $\alpha 4$ helix) (Figure 1). Following protein purification, time-dependent rates of chromate [Cr(VI)] reduction were measured, permitting a determination of maximal catalytic rates (V_{max}) and apparent affinities for chromate through measurements of the Michaelis-Menten constant (K_m) (Figure 2). In all cases, mutant enzymes exhibited 50% or greater decreases in maximal catalytic rates of chromate reduction (Table 1).

3.2. Relationship between FMN Positioning and Chromate Binding

From the available crystal structure of ChrR (3s2y.pdb), the FMN cofactor is held within a pocket near the surface through noncovalent interactions within individual subunits involving 12 electrostatic and 5 hydrophobic contact interactions that primarily involve loops 1 ($\text{G}^{14}\text{-SLRKASFN}^{22}$) and 4 ($\text{P}^{82}\text{EYNY}^{86}$) [8]. The ribityl phosphate group is positioned deep within the binding pocket, and is stabilized through a salt bridge with R^{17} in loop 1 between the $\beta 1$ sheet and $\alpha 1$ helix (Figure 1), which is highly conserved in other NAD(P)H-dependent FMN reductases [13,15,16]. The proximal S^{15} within loop 1 appears to stabilize this interaction, acting to assist in positioning the ribityl phosphate through the formation of a hydrogen bond. The stabilizing interactions between

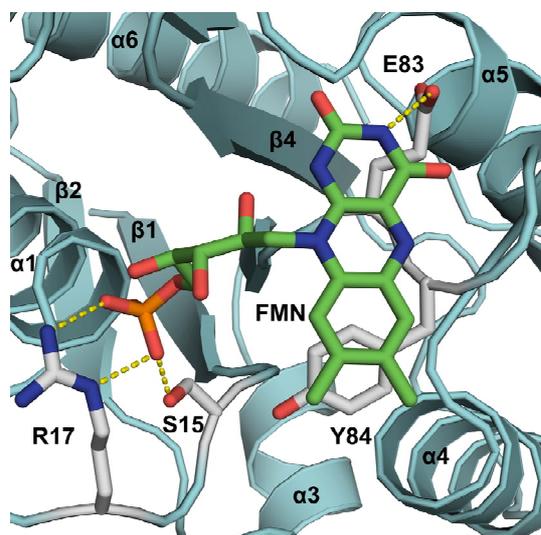


Figure 1. Mutant design for destabilization of the conformation of FMN within the binding cleft of Gh-ChrR Monomers. FMN and side chain under investigation are depicted as sticks, highlighting electrostatic interactions (dashed yellow lines). Secondary structural elements of Gh-ChrR are shown schematically (blue). Site-specific mutations (underlined) were made in loop 1 (GSLRKASFN²²) between the $\beta 1$ sheet/ $\alpha 1$ helix and loop 4 (PEYNY⁸⁶) between the $\beta 3$ sheet/ $\alpha 4$ helix structural motifs, which respectively stabilize the ribityl phosphate or isoalloxazine ring of FMN near the surface of individual subunits in the crystal structure of Gh-ChrR (3s2y.pdb).

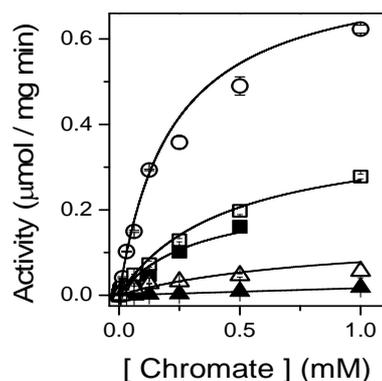


Figure 2. Inhibition of chromate reductase activity upon destabilization of bound FMN. Catalytic rates of reduction measured as a function of chromate, using absorbance changes of NADH, for wild-type Gh-ChrR (open circles), mutations in loop 1 (GSLRKASFN²²) between the $\beta 1$ sheet/ $\alpha 1$ helix (S15A, closed squares; R17A, closed triangles), or mutations in loop 4 (PEYNY⁸⁶) between the $\beta 3$ sheet/ $\alpha 4$ helix structural motifs (E83A, open squares; Y84A, open triangles). Catalytic rates were measured at 37°C in 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.1 mM NADH, and 5 μ M Gh-ChrR.

Table 1. Kinetic parameters for chromate reductase activity*.

ChrR variant	V_{max} (μ mol/mg·min)	K_m (mM)
wild-type	0.78 ± 0.07	0.22 ± 0.03
S15A	0.24 ± 0.04	0.31 ± 0.08
R17A	<0.02	N.D.
E83A	0.39 ± 0.03	0.4 ± 0.04
Y84A	0.1 ± 0.1	0.5 ± 0.2

*Parameter values calculated from nonlinear least squares fits of enzyme activity (Act) to the Michaelis-Menton equation, where $Act = (V_{max} \times [chromate]) / (K_m + [chromate])$, where $n = 3$.

loop 1 and the ribityl phosphate are expected to be important for catalysis, as the isoalloxazine ring of the FMN sits within a more hydrophobic cleft, whose positioning is likely dependent on the correct positioning of the ribityl phosphate. Consistent with this expectation, mutations S15A and R17A respectively inhibit overall rates of chromate reduction by 70% and >97%.

Mutations E83A and Y84A respectively affect a hydrogen bond with the N3 nitrogen on the isoalloxazine ring and a π - π stacking interaction that together positions the isoalloxazine ring in the binding cleft, resulting in a 50% and 87% inhibition in the rates of chromate reduction. These latter results suggest that the orientation of the isoalloxazine ring of FMN in the binding cleft plays an important role in modulating the overall rates of chromate reduction. This latter result is consistent with the putative assignment of the electron density proximal to the isoalloxazine ring of FMN in the crystal structure as a metal binding site capable of binding chromate [8].

3.3. Proposed Structural Coupling between FMN Binding Cleft and Chromate Binding Site

As we have already described (see above), loop 4 (P⁸²EYNY⁸⁶) between the $\beta 3$ sheet and $\alpha 4$ helix provides the ligands for the isoalloxazine ring of FMN. This is significant, as loop 4 also contributes to NADH binding through the involvement of N⁸⁵ [8] (**Figure 3**). Further, R¹⁰¹ in the $\alpha 4$ helix within this conserved FMN binding motif serves as a ligand to coordinate an anion (*i.e.*, chloride) in the crystal structure of ChrR, and has been suggested to act as a primary ligand for chromate ion binding [8]. These latter suggestions are consistent with the experimental data, demonstrating similar decreases in catalytic rates upon mutations in either loop 1 or 4 that modify the positioning of the FMN cofactor, and suggest the presence of long-range structural effects through the FMN binding pocket that affect reduction at the chromate binding site. Further, such mutations may couple to modify NADH binding (not tested here), which may

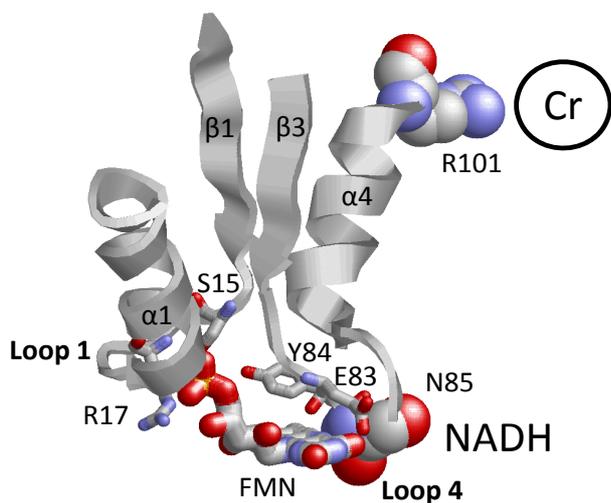


Figure 3. Proposed structural model between fmn binding cleft and amino acid side chains implicated in chromate reduction and NADH binding. Depicted are relative positions of FMN (wireframe image) in loop 1 (GSLRKASFN²²) between the $\beta 1$ sheet/ $\alpha 1$ helix and loop 4 (PEYNY⁸⁶) between the $\beta 3$ sheet/ $\alpha 4$ helix structural motifs (E83 and Y84 are shown). Mutated FMN ligand S15, R17 E83, and Y84 are shown in wireframe representation. Structural coupling between NADH and chromate binding is proposed through FMN binding cleft, involving NADH binding ligand N85 in loop 4 and chromate binding ligand R101 in $\alpha 4$ helix.

disrupt the NADH-dependent reduction of chromate. Such an allosteric coupling between the FMN binding cleft and NADH may represent part of a conformational switch that enables efficient NADH binding and subsequent release of NAD^+ following the reduction of FMN during the normal turnover of ChrR in the presence of natural substrates (*i.e.*, quinones); this mechanism may not apply in the NADH-dependent reduction of chromate. In this latter case, the ability of chromate to bind within this natural FMN binding cleft suggests design features that may further optimize chromate reductase activity involving, for example, site-specific mutations that enhance chromate binding while having minimal impact on FMN or NADH binding.

3.4. Conclusion and Future Directions

In summary, our results demonstrate substantial reductions in catalytic activity for four mutant enzymes involving ligands within the FMN binding cleft that selectively disrupt interactions with the ribotyl phosphate or isoalloxazine ring, demonstrating an essential role for the positioning of the FMN cofactor for optimal rates of chromate reduction. The close positioning of NADH and the bound chromate suggests that the observed promiscuous chromate reduction activity does not directly involve the FMN cofactor. Rather, conserved structural elements within NAD(P)H-dependent FMN reductases may

provide a structural framework for the construction of improved enzymes with catalytic activities appropriate for bioremediation (e.g., chromate reduction). Future measurements should design mutants that selectively increase chromate binding without significantly affecting the structures of either FMN or NADH binding clefts, and explore the possible direct electron transfer between NADH and bound chromate.

4. ACKNOWLEDGEMENTS

We thank David Kennedy and Andy Plymale for their technical support. This work was supported by Laboratory Directed Research and Development Funds (LDRD #90001) at Pacific Northwest National Laboratory (PNNL). Financial support comes principally from The US Department of Energy's Office of Biological and Environmental Research. Battelle operates PNNL for the US Department of Energy under Contract DE-AC05-76RL01830.

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