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Abstract:
      Cr(V) is a carcinogen that oxidizes guanine aggressively to form spiroiminodihydantion (Sp) and guanidinohydantoin (Gh), both of which contain an unusual hydantoin moiety that cause G→T transversion mutations at a high rate. Endonuclease VIII (nei) can recognize and excise these oxidation products from DNA and is translated as one of five protein products of the Nei operon in *Escherichia coli* (*E. coli*). However, the functions of the other four proteins remain unknown. To address this gap in knowledge, we focused on one of the four that immediately precedes nei, the ybgL protein. Previous work by our group has suggested a role for ybgL *in vitro*. In the current study, we attempt to characterize the role of ybgL by oxidizing a synthetic oligo with Cr(V) and reacting the oxidized oligo with ybgL in the presence of different potential cofactors. Due to the presence of hydantoin moieties within the DNA, we modeled the ybgL protein to the Hydantoinase B class of enzymes, which recognize the hydantoin moiety. This study will attempt to elucidate the role of an uncharacterized protein in excising oxidation lesions caused by chromium toxicity.

Introduction:
      The genotoxicity of chromate species is due to the formation of high valent chromium species - especially Cr(V) - that can directly oxidize DNA. Some chromate species, namely Cr(V), can also activate oxidant-sensitive fluorescent dyes in a similar manner to reactive oxygen species (ROS) (1).
      Sugden and Martin (2002) used the Cr(V)-Salen complex to introduce lesions in synthetic oligonucleotides. However, these lesions were not the common guanosine oxidation product 8-oxo-2'-deoxyguanosine (8-oxo-G) generated by ROS, but rather the further oxidation products spiroiminodihydantoin (Sp) and guanidinohydantoin (Gh), both of which contain hydantoin moieties (Fig. 1). These two DNA lesions halted polymerase and created G→T transversion mutations at a higher rate than 8-oxo-G, indicating their increased carcinogenic potential (2).
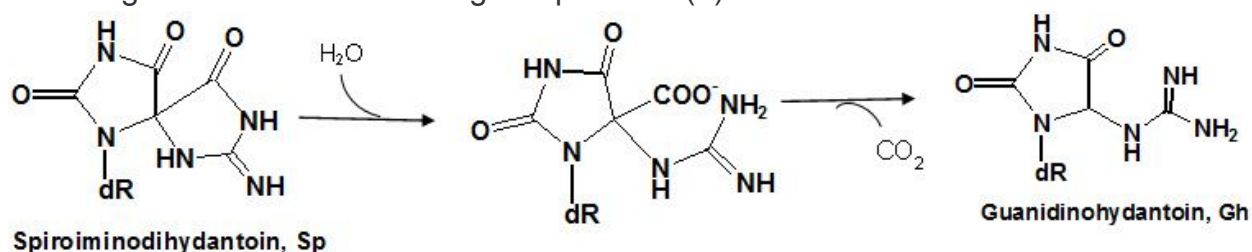

Figure 1. Mechanism of Gh formation from Sp, an oxidation product of 8-oxo-G.

      The question remained as to whether or not these lesions could form *in vivo*. To address part of this question, Cr(VI) was reacted directly with the intracellular reductant ascorbate and duplex DNA. They found that Sp was present in DNA at a concentration

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roughly 20 times that of 8-oxo-G, indicating that it is preferentially formed under hypothetical intracellular conditions (3).

To further this work and confirm the formation of Sp and Gh *in vivo*, Hailer *et al.* (2005) exposed Nei-deficient *E. coli* to chromate. Nei recognized the Sp lesion and is found on an operon that includes three other proteins of unknown function: YbgJ, YbgK, and YbgL. *E. coli* lacking the Nei enzyme exhibited significant growth inhibition in the presence of chromate. Mass spectral analysis of the genome of Nei-deficient *E. coli* revealed that Sp had formed *in vivo*, the first time this had been demonstrated (4). Interestingly, *E. coli* lacking the YbgL gene were unable to form endogenous Gh in response to chromium treatment (Whitfield, in preparation, 2016), suggesting that YbgL is necessary to convert Sp to Gh *in vivo* (Fig. 1).

YbgL is a member of the Lamb/Ycsf superfamily of proteins, which are so named because of their homology to LamA, an *Aspergillus* enzyme that hydrolyzes cyclic lactam molecules such as 2-pyrrolidone (Katz *et al.* 1989). Due to the hydantoin chemical scaffold of the Cr(V)-induced lesions Sp and Gh, we are investigating whether YbgL (and the LamB/YcsF superfamily to which it belongs) perform similar chemistry to the hydantoinase and cyclic amidohydrolase superfamily of enzymes. We wish to investigate whether YbgL is capable of hydrolyzing hydantoin-containing DNA lesions to Gh. Specifically, we wish to investigate whether YbgL is required for the hydrolysis of Sp to Gh (Fig. 1).

Materials and Methods:
Vector, Cell Lines, and Induction

The His-6-tagged wild type YbgL gene was placed in a pET26b expression vector that was under the control of the lac promoter. This vector was electroporated into BL21 DE3 *E. coli* (phenotype: F-ompT hsdS(rB- mb-) gal dcm λ(DE3)), which was grown in 50 µg/mL kanamycin in LB media. YbgL expression was induced using 1 mg/mL IPTG at an OD_{600} between 0.09 and 0.13 and was allowed to grow for 4-6 hours. Cells were pelleted at 4500 RPM, 4°C, for 10 min. The pellet was weighed and frozen at -20°C.

YbgL Purification

BL21 cell pellets were lysed using 1.2 mL NPI-10 (150 mM NaCl, 50 mM Na2HPO4, pH 7.52, 10 mM imidazole), 2 mg lysozyme per 0.2 g cell pellet, 200 µL 1 M $MgSO_4$, 8 µL Benzonase per 0.2 g cell pellet (Novagen), and 10 µL protease inhibitor cocktail (CalBioChem). The cell lysate was centrifuged at 15,000g and 4°C for 10 minutes to collect solid cell debris while leaving WT YbgL suspended in the supernatant.

Nickel-chelated nitrilotriacetic acid (NTA) resin (Qiagen), which was stored in 20% EtOH, was equilibrated with NPI-10. Cell lysate supernatant was added to the resin, and the mixture was rocked at 4°C for one hour to bind the 6-His tag of YbgL to the nickel on the resin.

The resin was then poured into a column and the flow-through was collected for SDS-PAGE analysis. Three 5 mL washes of NPI-20 (same as above, but with 20 mM imidazole) were conducted to remove any low-affinity endogenous proteins other than

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YbgL. Three 2 mL batches of NPI-500 (500 mM imidazole) were conducted to elute the YbgL protein. The purity of WT YbgL was determined using 15% SDS-PAGE (Fig. 2).

After purification of YbgL, the elution fractions were set to dialyze overnight with stirring at 4°C (150 mM NaCl, 50 mM NaH_2PO_4, 5% glycerol, 1 mM mercaptoethanol). Concentration of the purified protein was determined via Bradford Assay, using bovine serum albumin as a standard.
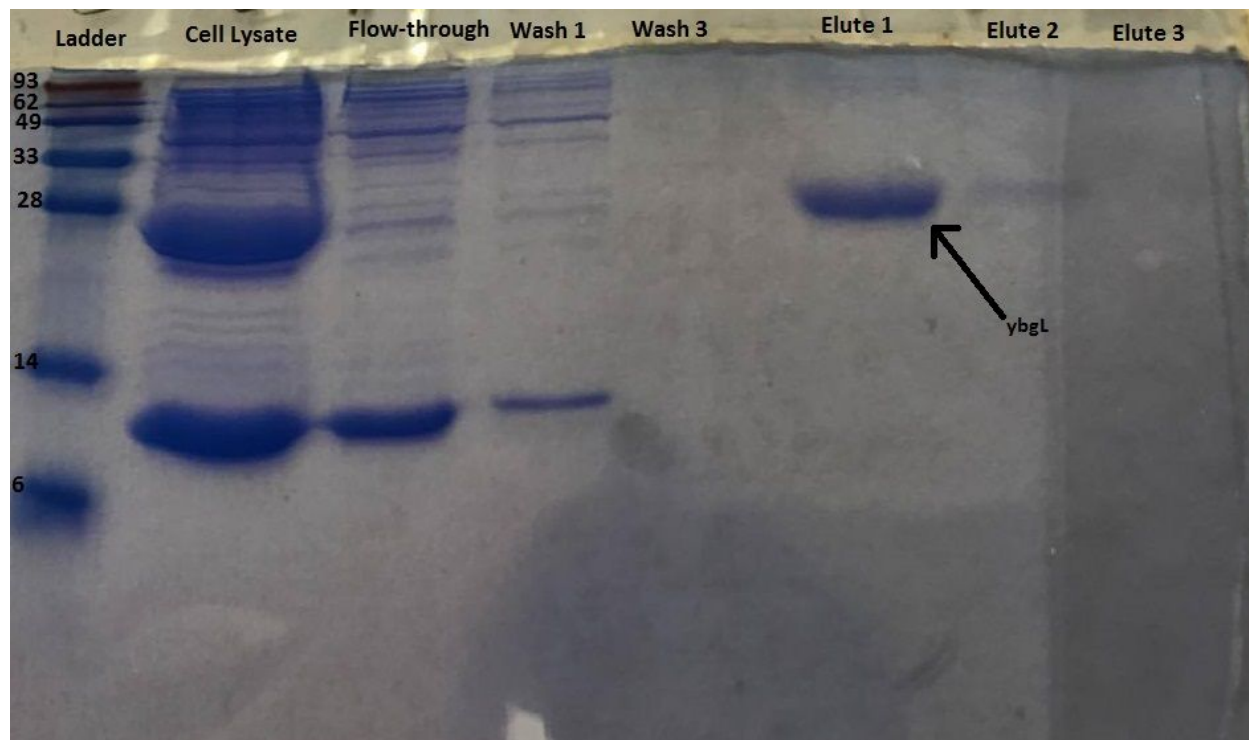

Figure 2. 15% SDS-PAGE of the chromatography fractions from Ni-NTA purification of WT YbgL.

Cr(III)-Salen Reaction

The reaction of chromium (III) chloride with the N,N'-bis(salicylidene) ethylenediamine (Salen) ligand was carried out as described by Coggon *et al.* ((5) and Sugden *et al.* (6). Cr(III)-Salen was synthesized as the hexafluorophosphate salt. Reddish-brown needles of Cr(III)-Salen were crystallized from water (Fig. 3).

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Figure 3. Cr(III)-Salen crystals, synthesized as described by Sugden *et al.* (2001).

Oligo Oxidation

Cr(V)-Salen was synthesized by adding 5 mg of the Cr(III)-Salen complex described above to 5 mg of iodosylbenzene in 500 uL of acetonitrile, as described by Sugden *et al.* (6). This reaction was incubated at room temperature for 30 minutes prior to its addition to the oligo reaction.

Oxidation was carried out similar to the procedure described by Sugden, Campo, and Martin (6). Specifically, we used the Cr(V)-Salen complex created above to oxidize 100 uM of a 8-oxo-G-containing synthetic oligo (5'ACCAGCGXCCGCACCAGT, where X is 8-oxo-G) or a 8-oxo-G-containing synthetic fluoro-oligo (5'(7-Methoxycoumarin) (C6-NH) ACCAGCAGCGXCCGCACCAGTG) with 20 uM Cr(V) at a pH of 8.05 to favor Sp formation. Both oligos were purchased from TriLink Biotechnologies. Reactions were conducted in 100mM K_2HPO_4 for 30 min. at room temperature. Control reactions were carried out using the same amount of acetonitrile as the oxidation reaction to control for solvent effects.

HPLC Separation

The oligonucleotide oxidation products were separated using a Dionex Nucleopac PA-100 4 x 250-mm anion exchange column using 10% acetonitrile with a gradient of 10-100% 1.5 M Ammonium Acetate in 10% acetonitrile pH 6.01 over 20 min.

Structural Modeling

Two putative lactam utilization enzymes in the LamB/YcsF family were structurally analyzed using PyMOL for similarities to YbgL. An overlay of one subunit from each of three LamB/YcsF proteins was created using PyMOL: YbgL (PDB: 1xw8), and two putative lactam utilization proteins from *Pyrococcus horikoshii* (PDB: 1v6t) and *Thermos thermophiles* (PDB: 2dfa). The tri-histidine site in YbgL (H57, H106, and H3)

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was modeled against those found in D-hydantoinase (PDB: 1NFG) and oxalate oxidase (PDB: 2ete), which both bind manganese cofactors.

Results:

      Oligonucleotides containing Sp or Gh were synthesized and purified (Fig. 4). Following analysis by mass spectrometry, these will be used to measure the binding of YbgL to each of the modified oligonucleotides. Structural modeling indicated a structural similarity of YbgL to the active site of hydantoinase molecules as well as the other manganese-containing enzymes examined in this study (Figs. 5, 6).

      Furthermore, preliminary observations have indicated that YbgL requires manganese to dimerize in a stable manner. This is indicated by the tendency of YbgL to precipitate out of solution in the absence of manganese (not shown). This observation, in conjunction with the structural modeling results, provides evidence for the involvement of manganese as a cofactor in the dimerization and enzymatic activity of the YbgL protein. To verify this initial result, our group will grow the bacterial expression hosts in the presence of manganese to allow this cofactor to be incorporated *in vivo* prior to the purification and concentration of the YbgL protein.

Discussion:

      Because of the structural similarities between the cofactor binding site of YbgL and the proteins mentioned above, our group will attempt to grow expression hosts in manganese, since we postulate that this cofactor may be required for the stability and activity of YbgL. As is shown in the chromatogram (Fig. 4), not much Sp was created upon initial oxidation reactions. To increase the efficiency of this reaction and produce more of the hypothetical substrate of YbgL, future experiments will be conducted under softer oxidization conditions (ie: higher pH) in order to favor Sp formation.

      The next step in this project will be to demonstrate binding of YbgL to oxidized DNA lesions in vitro. To provide more insight into the structure and function of YbgL, x-ray crystallography studies will be conducted to determine whether the tri-histidine cluster of YbgL requires a metal cofactor, particularly manganese, to stabilize and form and active protein.

      The work presented above has laid the foundation for future experiments to prove the role of YbgL as a hydantoinase enzyme that acts on oxidized guanine lesions produced as a result of chromium toxicity. This role will categorize YbgL as a base excision repair enzyme, thus identifying this previously-uncharacterized protein as important to repairing oxidatively-damaged DNA. Understanding the function of YbgL *in vitro* will shed light on the mechanism of chromium toxicity as well as the cellular safeguards against the conception of chromium-based cancer. This work will advance the understanding of the nuances of chromium toxicity, and will provide insight into the mechanisms by which chromium lesions can give rise to cancer, thus making prevention and treatment more effective.
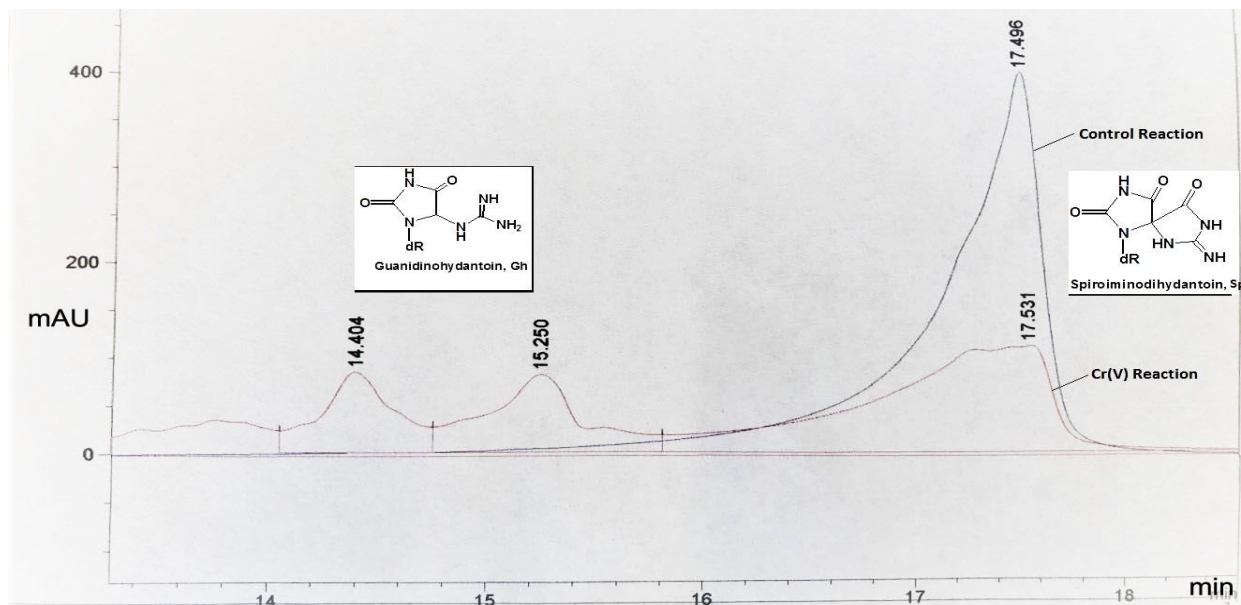
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Figure 4. Chromatogram of the separated oxidation products following oligo oxidation by Cr(V)-Salen. Structures of the expected products are listed above the time points where they hypothetically elute.
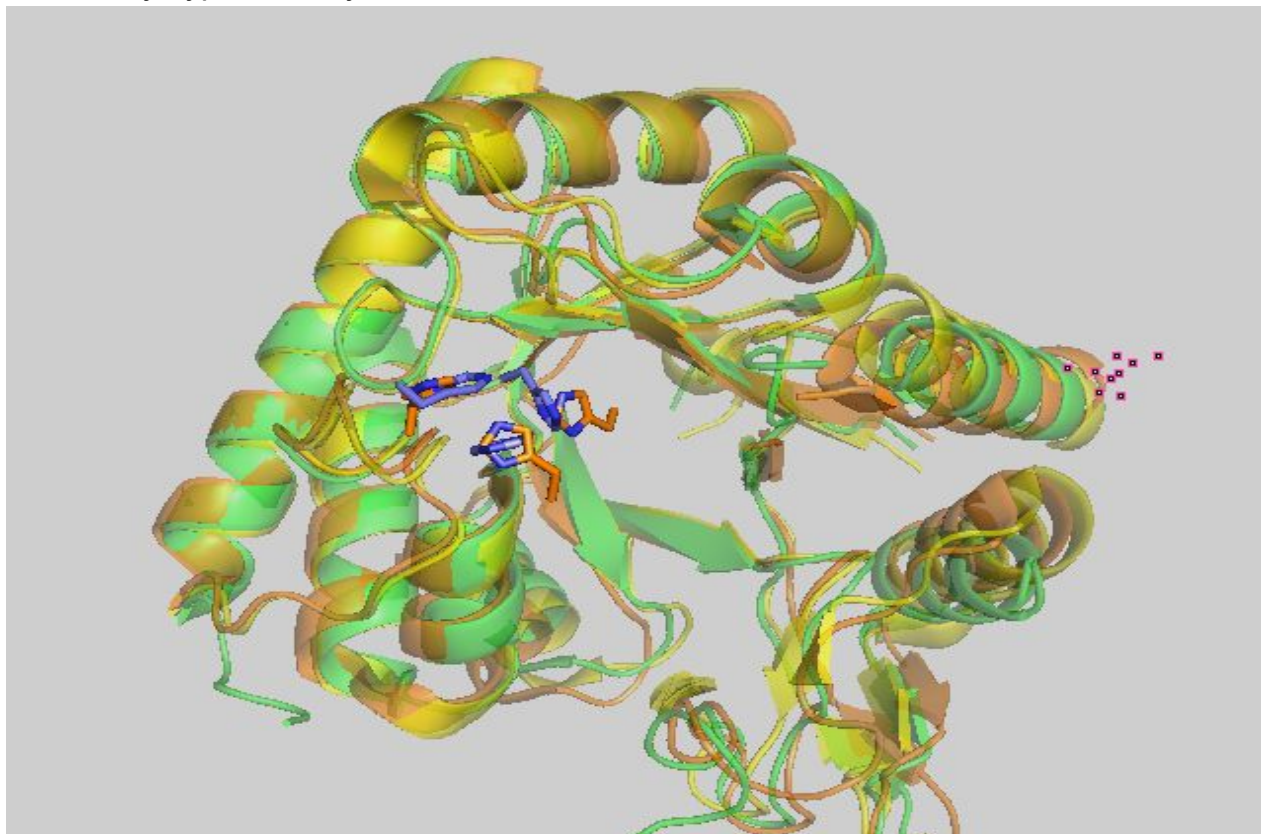

Figure 5. Above is an overlay of one subunit from each of three LamB/YcsF proteins: YbgL (green; PDB: 1xw8) and two putative lactam utilization proteins from *P. horikoshii* (yellow; PDB: 1v6t) and *T. thermophiles* (orange; PDB: 2dfa). Note the histidine cluster in the active site.
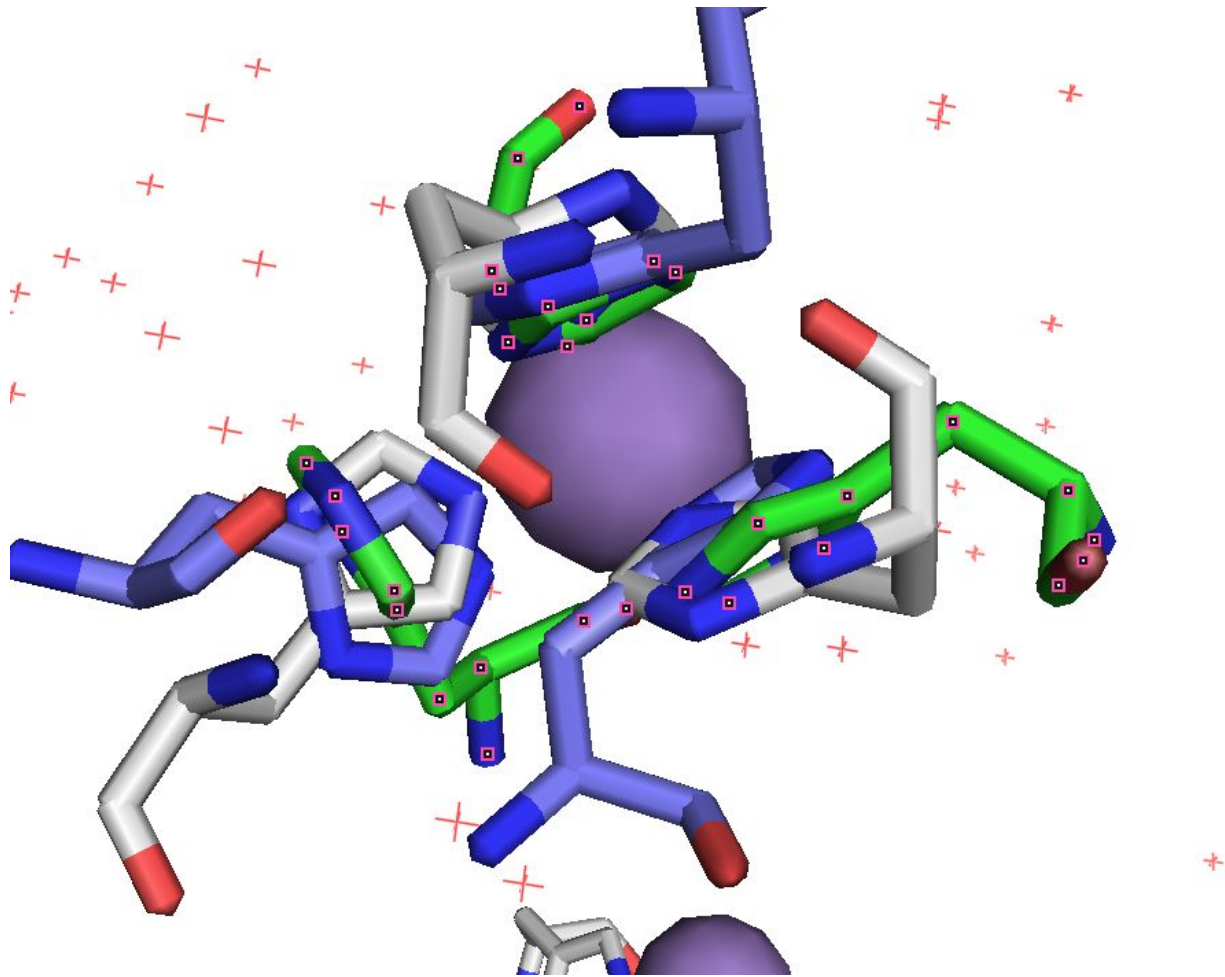
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Figure 6. The tri-histidine cluster present in YbgL (H57, H106, and H3) mirrors those found in D-hydantoinase (PDB: 1NFG) and oxalate oxidase (PDB: 2ete), which both bind manganese cofactors.

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