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In silico Structural Characterization of Rv3473c from *Mycobacterium tuberculosis* H37Rv: Potentials in Drug Development

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Authors' contributions

This work was carried out in collaboration between all authors. Author VT designed research. Author NI performed experiments. Authors NI and VT analyzed data, authors NI, GA and VT wrote the paper. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Large proportion of *Mycobacterium tuberculosis* H37Rv proteome still needs to be characterized especially peroxidase proteins as drug targets. There is twenty (20) known peroxidases that have been annotated in the genome. Among these Rv3473c has been studied by us using suitable *in silico* methods.
Methodology: The multiple sequence alignment of Rv3473c with PDB IDs 1BRO (277 aa), 1A7U (277 aa) and 1BRT (277 aa) indicates that the protein has well defined peroxidase motif. The 3-D molecular model of Rv3473c was generated using 1BRO, 1A7U and 1BRT as templates.
Results: Structural characterization indicates that it has the 'perhydrolase motif –G-X-S-X-G-, and

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the catalytic triad consists of the residues Ser73, His236 and Ser208. In addition, it has been found to contain the residues like Ser73, Phe77, Leu33, His5, Gly8, and among these Ser73 is the probable halide (bromine) binding site. Charge distribution analysis of active site indicates positively charged pocket near Ser208 of the catalytic triad whereas His236 remains within the negatively charged region. Ser73 of the 'perhydrolase motif' lies in the positively charged region of Rv3473c, however, another part of this motif is present in the hydrophobic region of the protein. Rv3473c uses H_2O_2 as nucleophile to form peracids. The presence of active site residues, oxyanion hole support the catalytic mechanism of Rv3473c as reported for other non-metal dependent bromoperoxidases.
Conclusion: Rv3473c, found out to be a non-metal dependent bromoperoxidase through *in silico* means, may prove its efficiency in near future in designing better drugs against tuberculosis after further research on it.

Keywords: Drug; molecular modeling; Mycobacterium; peroxidase; tuberculosis.

1. INTRODUCTION

It is strange that despite extensive efforts worldwide, 8.6 million people fell ill with Tuberculosis (TB) in the year 2012, and among them 1.3 million succumbed to death [1]. The causative organism of TB is the *Mycobacterium tuberculosis* (Mtb). It is an aerobic pathogenic bacterium which starts the infection usually in the lungs, and depending on the immune system of host, it may get eliminated or may remain latent in the body [2]. Till date, different antitubercular drugs are in use. Isoniazid (INH) is the major drug of TB because of highest Early Bactericidal Activity (EBA) [3]. INH is a pro-drug which gets converted into a biologically active form by a catalase-peroxidase (KatG) of *M. tuberculosis* [4]. In addition, INH acts against mycolic acid which is the major cell wall component of Mtb. KatG, heme peroxidase transforms INH into an isonicotinyl-NAD adduct, and this adduct inhibits two principal enzymes (InhA and KasA) involved in the mycolic acid biosynthesis [5,6].

Another study revealed that isonicotinicacyl-NADH binds to the active site of InhA [7]. Later the interaction between KasA and INH was implied by the discovery of a covalent complex of INH, KasA and its acyl carrier protein (AcpM) from isoniazid treated *M. tuberculosis* [8]. Pyrazinamide (PZA), is a synthetic pro-drug that is converted by the amidase (product of the *pncA* gene) of *M. tuberculosis* to the active form pyrazinoic acid [9]. It may be mentioned that the absorption of pyrazinoic acid into the cell occurs in an acidic environment and is a passive process that does not utilize cellular energy [10]. Another drug, Ethambutol (EMB) is an inhibitor of cell-wall synthesis that acts on the arabinosyl transferase (product of emb operon) which is involved in mycolic acid metabolism [11].

In the second line anti-tuberculosis agents, we may include different fluoroquinolones such as moxifloxacin (MFX), gatifloxacin (GTX), levofloxacin etc [12]. Among these the chemically related MFX and GTX are most active against *M. tuberculosis*, whereas levofloxacin slightly less active [13]. The site of action of fluoroquinolones is the DNA. They act on the gyrase and topoisomerase IV on DNA and converts them into ternary complexes, which results in blocking the movement of replication forks and transcription complexes [14].

Such chemotherapy and other prophylactic measures did help in reduction of incidence as well as eradication of TB for several decades. But now as a consequence of development of MDR strains of *M. tuberculosis*, Besides fluoroquinolones, injectables like aminoglycosides (amikacin and kanamycin) and capreomycin may be used in treating MDR (Multiple Drug Resistant) TB disease [15]. The incidence of synergistic diseases with TB like AIDS/HIV (which directly impairs the human immune system) [2], TB is again on the rise, which makes it really urgent to do extensive research in the designing of new anti-TB drugs.

In this respect we have explored various peroxidases of *M. tuberculosis* that might prove very useful in developing of new anti-TB agents [16]. The main reason behind this belief on peroxidase and in their anti-tubercular activity is because of KatG, which has a peroxidase part in it and plays a very crucial role in activating the major pro-drug of TB, *i.e.* isoniazid as described earlier [4]. Presently, around twenty numbers of peroxidases have been identified in Mtb (http://genome.tbdb.org/annotation/genome) [17] and characterization of these peroxidases may open up new frontiers in the development of better and stronger drug candidates against active tuberculosis. Most of these are yet to be

explored, and from this population of peroxidases we've chosen Rv3473c (having 261 amino acid residues) as potential candidate for enhancing effect of certain drug molecules on Mtb. It would possibly control further spread of the disease. Multiple Sequence Alignment (MSA) of Rv3473c indicates that it belongs to bromoperoxidase family and 3-D molecular models indicate several structural features required for bromoperoxidase activity. This presents a strong reason for its selection in this study over the rest of the peroxidases because we already have a very well characterized bromoperoxidase [18] and we may compare the same with the newly found one. Thus, it has been found to be a non-metallic bromoperoxidase. In this study we could obtain a 3-D model for a peroxidase that exists in the *M. tuberculosis* and might prove to be a handy tool to design better drug to control the incidence of tuberculosis.

2. MATERIALS AND METHODS

2.1 Multiple Sequence Alignment

The protein sequence of Rv3473c from *Mycobacterium tuberculosis* (Mtb) was retrieved from the TB genome database (http://genome.tbdb.org/annotation/genome). The protein is 261 amino acids in length and its corresponding gene is of 786 nucleotides in length. The protein sequence blasted into the NCBI non-redundant (nr) databank database using blastp [19,20] to identify homologous sequences. A multiple sequence alignment was performed with clustalW2 [21] to characterize Rv3473c from H37Rv. This MSA consisted of the sequences *S. aureofaciens* Bpo (Uniprot Accession: P29715); *S. aureofaciens* Clpo (Uniprot: Accession: O31168); *L. digitata* Vlpo (Uniprot accession number: Q4LDE6); *S.* sp. str. CC9311, VBpo {Uniprot: Primary (citable) accession number: Q0I6Q3}; *R. baltica* str. SH1 VClpo {Uniprot: Primary (citable) accession number: Q7UVW2} along with the Rv3473c (NP_217990). The parameters followed in clustalW2 are like Protein weight matrix – Gonnet, Gap open- 10, Gap extension- 0.20, Gap distances- 5, No end gaps- No, Iteration- None, Numiter- 1 and Clustering- NJ (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

2.2 Molecular Modeling and Structure Validation

The protein sequence was blasted into the NCBI protein databank (PDB) database using blastp to identify homologous structures. Among all the structures templates for molecular modeling were shortlisted on the basis of their degree of similarity and class of enzyme. Three proteins with PDB IDs 1BRO (bromoperoxidaseA2) (277 aa), 1A7U (chlorideperoxidaseT) (277 aa) and 1BRT (bromoperoxidaseA2 mutant M99T) (277 aa) were with sequence 98% homologous to the Rv3473c (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A graphics user interface easymodeller 4.0 for MODELLER 9v9 [22] was used to generate the 3D model of Rv3473c. The query Rv3473c and template sequences (1BRO, 1A7U and 1BRT) were used as input in the modeller and a total of 100 models were generated. These models were then subjected to energy minimization steps using the Swiss PDB viewer (www.expasy.org/spdbv/) [23,24]. Different models thus generated after every energy minimization step were validated with the help of various programmes like PROCHECK, ERRAT and Verify_3D. PROCHECK [25] programme was used to confirm the stereochemical properties and overall structural geometry of the protein structure. Verify_3D programme on the other hand determines the probability of attainment of a 3D model by the amino acid sequence of the newly generated model. It also compares the results with already existing structures in the protein structure database. ERRAT gives an overall quality factor that may aid to the reliability of the newly created protein model.

2.3 Protein Structure and Active Site Analysis

Active site is that part on the protein which remains conserved even in some distantly related enzymes and contributes to the substrate binding capabilities of the enzyme. Protein structure and active site analysis was performed by pymol-v1.3r1-edu-Win32.msi . Methodology for the same has been attained from the web link http://pymol.org/ep/ after online registration on the pymol website (http://pymol.org/educational/) [26].

It may be mentioned that all the *in silico* studies have been carried out in a desktop computer with the specifications as follows: Microsoft Windows XP, AMD Phenom(tm) II X3 710 Processor, 2.60 GHz HD and 3.25 GB of RAM. The manufacturer of the system is HCL.

3. RESULTS AND DISCUSSION

3.1 Rv3473c is a Non-metallic Bromoperoxidase

Multiple sequence alignment of Rv3473c with *S. aureofaciens* Bpo; *S. aureofaciens* Clpo; *L. digitata* Vlpo; *S*. sp. str. CC9311, VBpo; *R. baltica* str. SH1 VClpo was performed with CLUSTALW2 server (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to identify and characterize the proteins. Sequence alignment analysis indicates that Rv3473c is a perhydrolase as evident from the presence of the 'perhydrolase motif (-Gly-X-Ser-X-Gly-) as reported in other haloperoxidases [27] (Fig. 1). A multiple sequence alignment of Rv3473c with metal dependent or independent haloperoxidases indicates a sequence similarity of 43.29% with metal independent group and a 7.66% similarity with metal dependent haloperoxidases. In addition metal binding loop is completely absent in Rv3473c indicating it as non-metallic haloperoxidase present in mycobacterium genome (http://genome.tbdb.org/annotation/genome/tbdb/FeatureSearch.html). Non-metal dependent haloperoxidases can be grouped in 3 different families depending on the presence of specific residues for catalysis of chloride, iodide or bromide peroxidation [28]. A multiple sequence alignment with different class of haloperoxidases was carried out to characterize the class of Rv3473c. Sequence alignment analysis indicates that Rv3473c has specific residues present in bromoperoxidase but does not have crucial residues required to catalyze peroxidation reaction against chloride or iodide. Hence, sequence analysis in Fig. 1 concludes that Rv3473c is a non-metal dependent bromoperoxidase present in mycobacterium genome and we will refer this protein as 'MtBpo' in following sections.

Fig. 1. Multiple sequence alignment of Rv3473c with other halide peroxidase

3.2 Quality of Modeled Structure

In case of MtBpo, PROCHECK displayed 83.9% residues in most favoured regions, 13.3% in additional allowed regions, 2.3% in generously allowed regions and 0.5% in disallowed regions (Fig. 2A). Overall quality factor is 83.794 for the newly obtained model (Fig. 2B) while that for the template is 99.627.In this model 88.55% of the residues had an averaged 3D-1D score (Fig. 2C), which is very close to 100% of the template 1BRO confirming the good quality of the model (MtBpo).

3.3 Structure of MtBpo

3.3.1 Over all structure

As evident from earlier studies that peroxidases are important for development of new anti-tuberculosis drugs, and in this process it was very important to know the three-dimensional structure of the newly obtained model (MtBpo). MtBpo is a globular protein and belongs to the alpha/beta family (Fig. 3A). The modeled structure has 13 alpha (α) helices, 5 beta (β) sheets and rest is the unstructured loop region. The 5 β strands are lying parallel to one another and present in the centre of the protein. The topology of the MtBpo indicates that the β trands are connected to one another through different α helices.

(Fig. 3B). The strands 1-2 are connected by the helices A and B; 2-3 are connected by the helix C; 3-4 by D,E,F,G and H, and the strands 4-5 are connected by the helix K. From topology it could also be found that MtBpo is showing more helical and less β-sheet domains than 1BRO. On percent basis it may be stated as 42.11% of β strands, 57.89% of α helices in 1BRO, and 27.78% of β strands, 72.22% of α helices in MtBpo. As presented in Table. 1, this model is having the 'perhydrolase activity motif', i.e. –G-X-S-X-G-. It's also found that catalytic triad consists of the residues Ser73, His236, Ser208. In comparison to other enzymes, Ser 208 was present in the catalytic triad in place of Asp228. However, it will not affect the enzymatic reaction, as acidic component is of less importance as compared to the rest of the amino acids in the triad as far as catalytic activity of the enzyme is concerned. It has been reported for other α/β fold super family enzymes like thioesterase I, protease I and phospholipase L1 [29]. MtBpo has several charged grooves on it, some of which are positively charged (Blue), negatively charged (Red colored) and others are hydrophobic in nature (White coloured part) (Fig. 4).

3.3.2 Catalytic domain and active site

The catalytic triad consists of Ser73, His236, Ser208 (Table 1). In this the nucleophile Ser73 and the highly conserved His236 are present as usual, but it is devoid of regular acidic component which is generally either Asp or Glu. Instead of the acid component we could find an extra Ser residue at the 208th position. However, as stated earlier absence of Asp or Glu should not have a significant effect on the catalytic activity of the enzyme. One thing may be noted in this regard that at 209th position there's a Asp residue, but what role it may play is difficult to predict now. In MtBpo we may observe the presence of the 'perhydrolase activity motif' which is Gly71-Ala72-Ser73-Leu74-Gly75.

Table 1. Characteristics of Rv3473c

Sl. no.	Category	Characteristics	Rv3473c
1.	Biochemical parameters	Mol. Wt. pI	28.20KDa 5.148
2.	Structural elements	α/β hydrolase fold Catalytic triad Metal binding loop	Ala116 - Leu149 Ser73, His236, Ser208 Not Present
3.	Structural elements	α helices and β sheets	α helices = 72.22% β sheets = 27.78%
4.	Potential amino acids	Perhydrolase activity motif Peroxide binding Halide binding	Gly71, Ala72, Ser73, Leu74, Gly75 Leu74 (probable) Probable candidate could be Ser 73 at the active site.

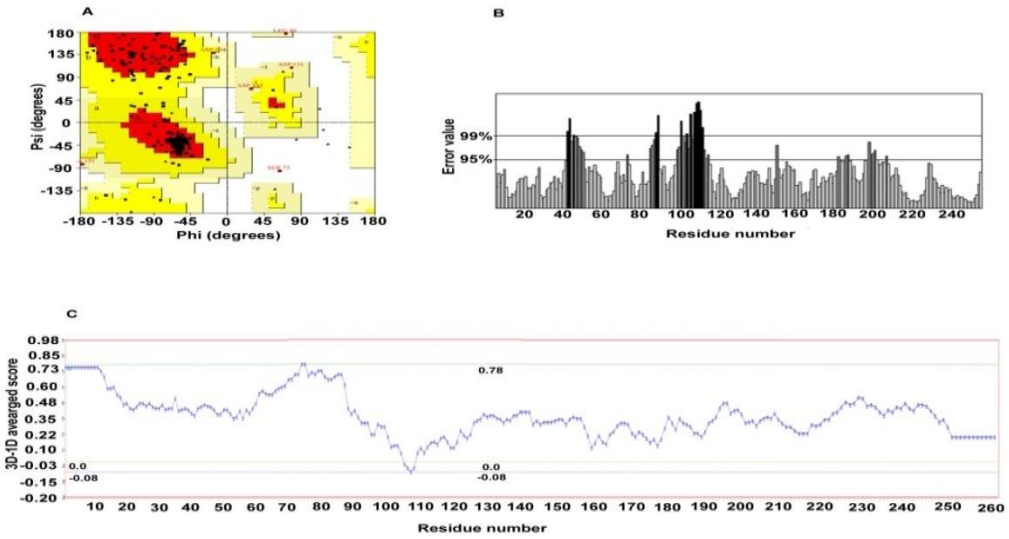

Fig. 2. Validation of modeled Rv3473c
(A) Ramchandran plot (B) errata plot (C) Verify 3-D

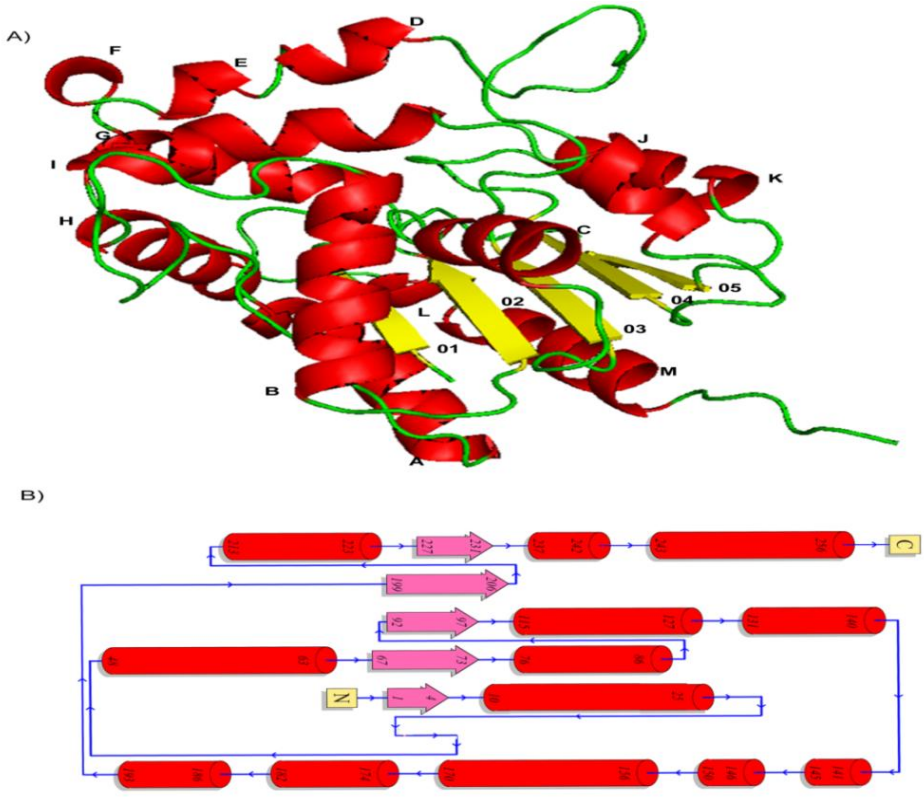

Fig. 3. Modeled structure of Rv3473c
(A) 3-D model of Rv3473c, (B) Topology of the modeled enzyme

In this motif the nucleophilic serine (Ser73) is found in the consensus sequence Gly-X-Ser-X-Gly as stated earlier. In MtBpo just after Ser73, we find Leu74. In the bromoperoxidase, 1BRO this position is occupied by Met99 and it reacts with H_2O_2 to get oxidized to methionine sulfoxide

[18]. Thus in case of MtBpo, without performing any laboratory experiment, it is difficult to predict which residue will actually react with H_2O_2. But absence of methionine or any other aromatic amino acid is also acceptable as reported in case of 1*H*-3-hydroxy-4-oxoquinoline 2,4-dioxygenase (Qdo) from *Pseudomonas putida* 33/1 and 1*H*-3-hydroxy-4-oxoquinaldine 2,4-dioxygenase (Hod) from *Arthrobacter ilicis* Ru¨61a [30]. One more point may be noted that unlike the bromoperoxidase (1BRO) where only one methionine residue (Met99) is present, in MtBpo we could find several Met residues at different positions like 1,80,103,115,186 and 237. As far as the active site pocket of MtBpo is concerned we find residues like Ser73, Phe77, Leu33, His5, Gly8 which are in corresponding positions with those in the template 1BRO. Ser73 is located between strand 2 and helix C (Nucleophile elbow); in case of 1BRO this part is further stabilized by a strong back bone hydrogen bond between Ser98 and T101, but for this bond to occur in MtBpo there is no amino acid residue present in the corresponding position of T101. Again, further stabilization of this region is provided by the buried salt bridge between Glu103 and Arg58 in 1BRO, but the corresponding amino acids in MtBpo are Phe77 and Leu33, and there cannot be a salt bridge between Phe and Leu due to presence of hydrophobic side chains. The Arg58 of 1BRO also connects helix C to the carbonyl oxygen of His30 at the end of strand 3 by a strong hydrogen bond, and in case of MtBpo we find leucine (Leu33) in place of Arg58 and it connects helix D to the His5 at the end of strand 1. Again in case of 1BRO the loop between the strand 3 and helix A, with the *cis*-proline Pro33 at its tip, provides the bottom of the active site pocket, whereas in MtBpo this region is occupied by the loop between the strand 1 and helix A, with the *cis*-glycine Gly8 at its tip.

3.4 Charge Distribution in the Catalytic Site

Ser208 of the catalytic triad remains in the positively charged part of the protein and His236 remains slightly towards the negatively charged part, while Ser73 lies slightly in the positively charged part (Fig. 4). It was observed that most part of the perhydrolase activity motif lies in the hydrophobic region of the protein, and rest lies in the positively charged zone (Fig. 4). The active site back entrance (Gly6, Ala72 and Pro146), to allow substrate entry in this part, lies within hydrophobic region of the protein (Fig. 4).

3.5 Proposed Mechanism of MtBpo

It uses H_2O_2 as nucleophile to form peracids catalysed by some hydrolases. The salient steps of catalysis are given in Fig. 5 and follow the mechanistic intermediates as described for other bromo-peroxidase [31]. MtBpo although has the perhydrolase activity motif (Gly71-Ala72-Ser73-Leu74-Gly75), yet it lacks the Asp in the catalytic triad. But as stated earlier it should not hamper the catalytic activity of the enzyme. Again, another important structural part in the active site is the oxyanion hole. The purpose of the oxyanion hole is to stabilize the oxyanion formed in transition state, after the nucleophilic attack. Hydrolases, such as chloroperoxidases (CPO-T, CPO-L) bromoperoxidase (BPO-A1), (-)-γ-lactamase and dihydrocoumarin hydrolase, likely use the same mechanism as L29P-PFE (*Pseudomonas fluorescens* esterase) for perhydrolysis of acetic acid since all of these catalysts contain a proline at the oxyanion loop [32]. As MtBpo is also a bromoperoxidase, it might also follow the same mechanism like the L29P-PFE as described. Another reaction scheme proposed for the bacterial cofactor-independent haloperoxidases that postulated the oxidation of a Met to methionine sulfoxide by H_2O_2 as the first step [29] of reaction may be abandoned as the Met residue is not conserved in these enzymes. As depicted in the flowchart, an acetyl-enzyme intermediate is involved in the mechanism. In the 1st diagram (of Fig. 5) from above, there's an enzyme-acetic acid complex.

The γ-hydroxyl group is a nucleophile in the Ser94 of the active site. This nucleophile attacks the carbonyl group of acetic acid and a tetrahedral intermediate (not shown) is formed via the curved arrows. After this, an acetyl-enzyme intermediate is formed when the tetrahedral intermediate collapses via the release of water. In the second diagram (of Fig. 5), another complex is formed because of binding of hydrogen peroxide with the already existing acetyl-enzyme intermediate. The hydrogen bonds in this case were donated by nitrogen hydrogen bonds of M95 and W28, which are called the oxyanion hole. A second tetrahedral intermediate is formed in the 3rd diagram (of Fig. 5) due to this nucleophilic attack of hydrogen peroxide on the acyl-enzyme [31].

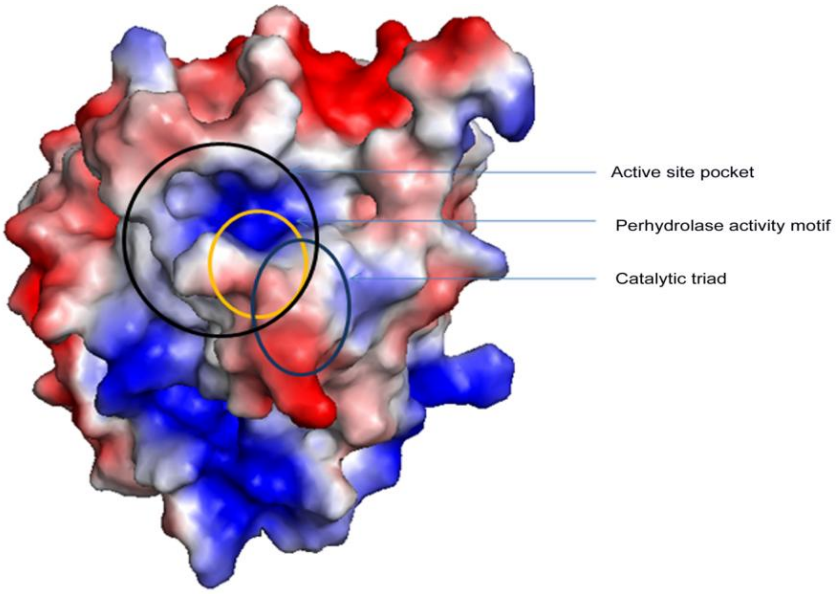

Fig. 4. Surface charge distribution of Rv3473c

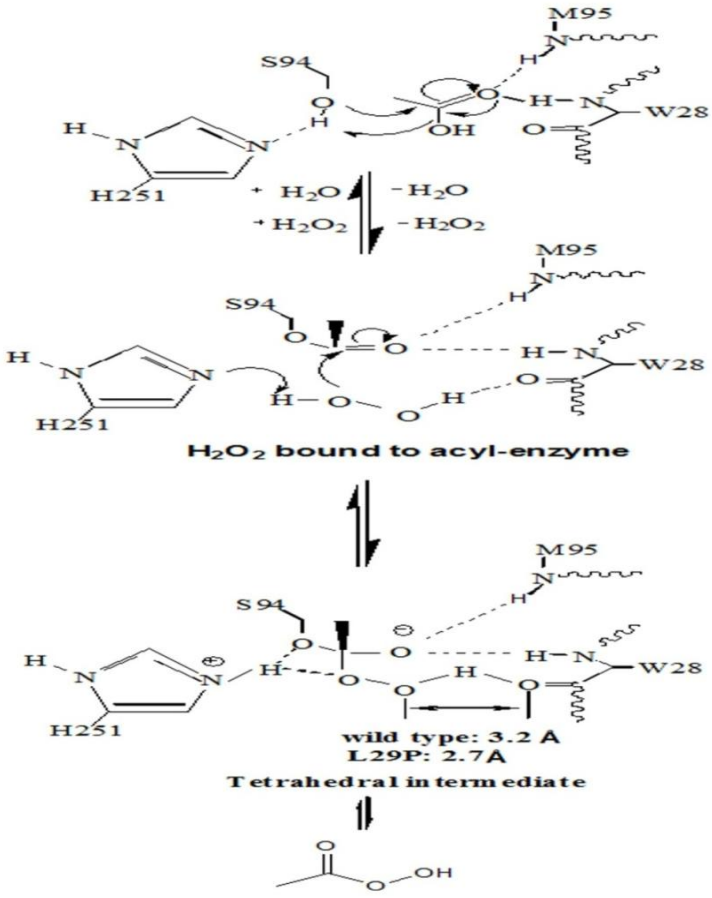

Fig. 5. Proposed catalytic mechanism of Rv3473c

4. CONCLUSION

In silico structural characterization indicates that Rv3473c has the 'perhydrolase motif –G-X-S-X-G-, and the catalytic triad consists of the residues Ser73, His236 and Ser208. The presence of active site residues, oxyanion hole support the catalytic mechanism of Rv3473c as reported for other non-metal dependent bromoperoxidases. From all these observations we now have a 3-D structure for Rv3473c which is competent enough to select as a drug target and its further use in developing a new and/or more efficient drug molecule against tuberculosis.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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