

Kinetic Analysis of the Amino Terminal End of Active Site Loop of Lactate Dehydrogenase from *Plasmodium Vivax*

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ABSTRACT

Objective: In this study, kinetic analysis was performed to understand the functional importance of the amino terminal of the active site of previously mutated *Plasmodium vivax* Lactate Dehydrogenase enzyme by mimicking *Toxoplasma gondii* I, II, *Eimeria acervulina* and *Eimeria tenella* LDH's.

Material and Methods: Mutant LDH genes were amplified by PCR and 6xHistag was added to the C-terminal of the enzymes. Then LDH enzymes are overproduced as recombinant in *E. coli* cells, purified by Ni-NTA agarose matrix and kinetic properties were analysed.

Results: Observing increase of K_m values of mutant enzymes showed that mutations in this place caused decreasing affinity of enzyme for its substrate. However k_{cat} values were about the same throughout all mutant proteins.

Conclusion: Sensitivity of the studied region emphasizes the significance of this site for drug design studies for both *Plasmodium* and some other *Apicomplexans*.

Key Words: Lactate dehydrogenase, *Plasmodium vivax*, antimalarial drug, site-directed mutagenesis, kinetic analysis

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Introduction

Malaria is an infectious disease caused by *Plasmodium* parasites which is transmitted to people via the bites of infected mosquito vectors belonging to the *Anopheles* genus. While malaria parasites spend some of their life cycles in the vectors, they need another host such as humans to complete their cycles. Five *Plasmodium* species cause disease in human: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and recently reported monkey malaria parasite *Plasmodium knowlesi* (1, 2).

Malaria shows a wide range of geographic distribution worldwide. About half of the world's population (3.3 billion people) is at risk of malaria. Each year about 250 million people are affected and nearly 1 million deaths occur, of which 90% are in sub-Saharan Africa (3). In the next decade, malaria infections are going to rise again in many regions of Africa, Asia and South America where it is already endemic. The major cause of this increase is the spread of resistance of the *Plasmodium* species to the conventional anti-malarial drugs (4). *Plasmodium falciparum* is one of the most deadly species of human malaria parasites and resists all known kinds of drugs except artemisinin (5). Chloroquine resistance of the most common parasite *Plasmodium vivax* is increasing gradually (5). Development of resistance to currently available

drugs and absence of any effective malaria vaccine has led to determining of novel drug targets (4).

Malaria parasites need high levels of energy to grow rapidly in the erythrocytes at the asexual growth stage. Absence of fully functional citric acid cycle directs parasites to gain most of the ATP energy from the glycolytic pathway for survival (6, 7). Thus, one of the first routes in the development of an antimalarial drug involves identification of vital parasite enzyme targets, and glycolytic enzymes have been selected as the new drug target candidates (4, 8).

Lactate dehydrogenase (LDH), the terminal enzyme of the glycolytic pathway, is a vital enzyme for the *Plasmodium* parasites and causes oxidation of pyruvate to L-lactate and provides turnover of NAD^+ required for the previous stages of glycolysis (8, 9). It has been shown that inhibition of the malarial LDH enzyme prevents the production of ATP and causes death of the *Plasmodium* parasites (8). *Plasmodial* LDH enzyme differs from its mammalian counterpart by means of kinetic parameters and also in molecular structure (10-12), so it becomes an attractive drug target candidate (10, 12-14).

Sequence of lactate dehydrogenase enzyme of *Plasmodium* and other *Apicomplexan* parasites share similar residues at the active site loop located between the 100th and 110th aminoacids. However the amino terminal end of the active site loop of lactate dehydrogenase between *Plasmodium* par-

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asites and some other Apicomplexan LDHs (*Toxoplasma gondii* LDH1 and LDH2, *Eimeria tenella*, *Eimeria acervulina* and *Theileria parva*) show amino acid exchanges (15). In this study, kinetic analyses were performed on previously mutated *Plasmodium vivax* LDH mutant enzymes by mimicking *Toxoplasma gondii* I, II, *Eimeria acervulina* and *Eimeria tenella* LDH's to understand the structural importance of the amino terminal end amino acids of the active site loop.

Material and Methods

Homology modelling

The LDH amino acid sequence in FASTA format of wild type *Plasmodium vivax* was obtained from NCBI (<http://www.ncbi.nlm.nih.gov>) with the accession number of 2A92_A. Amino acid changes on this template were made to obtain mutant proteins by using SWISS-MODEL, automated protein homology-modelling server (<http://swissmodel.expasy.org>). Constructed mutant protein models were expressed as Protein Data Bank (PDB) file format. PDB file of wild type PvLDH enzyme were downloaded from Protein Data Bank (<http://www.rcsb.org/pdb>) with the ID code 2A92. PDB files for every mutant protein models and also wild type one were analyzed for superimposition and visualized by Molsoft ICM-Pro 3.4-9a.

Bacterial strains, growth media, plasmid and enzymes

Escherichia coli JM105 strain {*supE endA sbcB15 hsdR4 rpsL thiD (lac-proAB)F' [traD36 proAB+ lacI^q lacZDM15]*} was used as a host for plasmids propagation and as an expression strain. Strains were cultured in double-strength yeast-tripton (2xYT) agar plate and broth media at 37°C. Ampicillin was used in the media in appropriate amounts (100 µg/mL) for the selection and growth of transformants (16). The *E. coli* plasmid vector pKK223-3 (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) was used for subcloning, expression and sequencing. *Pfu* DNA Polymerase (2.5 U/µL) was supplied by Fermentas (Lithuania), *EcoRI* (12 U/µL) and *PstI* (10 U/µL) restriction enzymes and T4 DNA Ligase (3 U/µL) were purchased from Promega (USA).

Addition of His tag to the mutant PvLDH genes

His tag was added by PCR amplification to the C-terminal of previously mutated (17) *P. vivax* enzymes. Primers used for amplification complementary to the forward and reverse strands were Pv7; 5'-GCC GAC CCG GAA TTC ATG ACG CCG AAA CCC AAA ATT GTG C-3' (*EcoRI* restriction site) and Pv15; 5'-TTT TCT GCA GTT AGT GAT GGT GAT GGT GAT GAA TGA GCG CCT TC ATC C-3' (*PstI* restriction site and 6xHis tag site). PCR was performed in a total of 50 µl volume containing 5 µL 10x *Pfu* Buffer, 5 µL stock dNTPs (10 mL of each 10 mM dNTPS and 10 mL of dH₂O), 2.5 µL of each 5' Primer (Pv7, 20 pmol/µL) and 3' primer (Pv13, 20 pmol/µL), 1 µL template mutant PvLDH DNA, 1 µL *Pfu* DNA Polymerase (2.5 U/µL) and 33 µL sterile dH₂O. The PCR was carried out at 94°C for 1.5 min, 50°C for 2 min. and 72°C for 2 min. for 30 cycles. Double-strand amplicons run on a 1% crystal violet stained agarose gel for preparative purposes and a parallel PCR product obtained using the same reaction was run on

the 1% ethidium bromide (EtBr) stained agarose gel for visualisation of the amplicons (18). The DNA was recovered by QIA quick Gel Extraction Kit (QIAGEN, Germany) following the electrophoresis.

Restriction digest, ligation and transformation

Both of the mutant DNA samples and pKK223-3 expression vector were digested by *EcoRI* and *PstI* restriction endonucleases. Digested vector and samples were mixed at the ratio of 1:1 and the mutant genes were ligated into digested expression vector pKK223-3. The reaction mixture contained 100 ng DNA (insert), 100 ng vector, 1 µL T4 DNA Ligase (3 U/µL), 1 µL 10X ligation buffer and XµL sterile dH₂O to a final volume of 10 µL. The ligation was set up at 4°C overnight in the thermal cycler (Eppendorf). The resulting recombinant DNA plasmids were transformed into CaCl₂ competent cells of *E. coli* JM105 according to Sambrook et al. (16) and plated onto the 2xYT agar plates supplemented with 100 µg/mL ampicillin and grown overnight at 37°C.

DNA sequencing

After transformation, the resultant colonies were screened by colony PCR for plasmid inserts using Pv7 and Pv15 primers. Plasmid DNAs were purified from the positive colonies using QIAprep Spin Miniprep Kit (QIAGEN) and then sequenced by lontek (Turkey) using internal primers to show the addition of the 6-His tag to the C terminal of the mutant genes.

All general methods were applied according to Sambrook et al. (16) unless otherwise stated.

Purification of the His tagged mutant PvLDH enzymes

Starting culture of *E. coli* JM105 containing recombinant plasmids were inoculated into yeast-tripton (2xYT) broth containing ampicillin (100 µg/mL) and grown at 37°C overnight. The culture was inoculated (2 mL) into 500 mL growth medium and the cultivation was performed until OD₆₀₀ reached 0.6. Expressions of mutant PvLDH enzymes were induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) with 1 mM final concentration and incubated overnight at 37°C while shaking at 200 rpm. Subsequently, protein purification was performed according to Shoemark et al. (19).

Following the incubation, cells were harvested by centrifugation at 5000 rpm for 15 min at 4°C and the pellet was suspended in 6 mL lysis buffer (50 mM Na₂HPO₄, 300 mM NaCl, 20 mM imidazole, 10% glycerol, pH 7.5). The cells were sonicated at 60% Amp. (9x10s pulses with 30s intervals) on ice and centrifuged at 14.000 rpm for 1 hour at 4°C to remove cell debris. Supernatant was collected and used in the following applications. Ni-NTA agarose matrix was equilibrated with the lysis buffer prior to loading the sample. The supernatant was applied to the column and flow through was collected. The column was washed with 40 mL wash buffer (50 mM Na₂HPO₄, 300 mM NaCl, 20 mM imidazole, 10% glycerol, pH 7.5) which is the same as lysis buffer and fractions were collected. Proteins bound to the column were eluted using 10x of 1 mL elution buffer (50mM Na₂HPO₄, 300 mM NaCl, 250 mM imidazole, 10% glycerol, pH 7.5) (19). The purity of protein fractions were checked by both SDS-PAGE (20) and by scanning the

protein at 260-320 nm by UV-visible spectrophotometer. Protein concentration was determined by using the absorbance value obtained at 280 nm by UV-visible spectrophotometer.

Western blot analysis of mutant proteins

The primary antibody that was raised against active *P. falciparum* LDH in New Zealand white rabbit was used in the assays (13). The secondary antibody, goat anti-rabbit IgG, was purchased from the Bio-Rad, UK. Protein samples were run on the SDS-polyacrylamide gel prior to blotting (20). Proteins were transferred to nitrocellulose membranes electrophoretically after the SDS-PAGE for western blotting. The membrane was blocked in PBS Tween 20 containing 5% skimmed milk powder overnight at 4°C. After the blocking, the membrane was washed in PBS Tween 20 for 10 minutes and then incubated in the blocking buffer with 1:1000 diluted primary antibody at room temperature for 1 hour on a shaker. After the incubation the membrane was washed three times in PBS tween 20. The goat anti-rabbit IgG conjugated to peroxidase was diluted 1:12000 in 5% PBS Tween 20, and the washed membrane was incubated in this solution at room temperature for 1 h. Later, the membrane was washed twice in PBS Tween 20, once in PBS, and once in H₂O. Finally, the filter was incubated in 60 mg 3.3 diaminobenzidine (DAB), 100 mL PBS, 100 mL H₂O₂ for 30 seconds and rinsed in H₂O (13).

Kinetic analysis of the mutant PvLDH enzymes

Molecular masses and extinction coefficients of mutant PvLDHs were calculated by using web based the "Peptide Property Calculator" (<http://www.basic.northwestern.edu/biotools/proteincalc.html>) programme. Steady-State kinetic parameters of the mutant enzymes were determined by following the rate of absorbance change at 340 nm using the UNICAM UV/Visible spectrophotometer. Enzyme assays were performed in 50 mM Trizma Base/KCl kinetic buffer, pH 7.5, containing 200 µM NADH at different concentrations of pyruvate (20 µM, 60 µM, 180 µM, 340 µM, 1.6 mM, 4.8 mM, 14.48 mM, 20.4 mM, 29 mM) at 25°C. Data were used to determine K_m and V_{max} values and using Grafit 3.0 and then the values were used to calculate k_{cat} (21). Kinetic analysis was performed without removing 6xHis tag from the N- terminal of the enzyme which had no effect on the kinetic constants (22).

Results

Sequence alignment of PvLDH to other target LDHs

The N terminal sequence of the active site loop placed between positions 100-108 of wild type PvLDH enzyme was compared to the same region from the previously mutated PvLDH enzymes (Table 1) (17). Alignment results indicated the terminal sequence of the active site of PvLDH is slightly different from other Apicomplexans LDHs which differ from 2 or 3 residues. Amino acid exchanges made on the PvLDH in the previous study to mimic *Toxoplasma gondii* I LDH (TgILDH) were F100L, A103V and S108P; for *Toxoplasma gondii* II LDH (TgIIILDH) F100L and A103V, for *Eimeria tenella* LDH (EtLDH) F100I and P105A; for *Eimeria acervulina* LDH (EaLDH) were F100I and A103I (17).

Addition of His tag to the mutant PvLDH genes

Amplification of the mutant LDHs were performed by using specific primers that had a His tag to enable purification of the protein by affinity purification and amplicons run on a 1% Ethidium Bromide (EtBr) stained agarose gel to confirm the presence of the correct band (Figure 1). EtBr stained gel was only used to identify the molecular weight of the amplified genes. Gel result indicated the amplicons were about 1 kb pair long. This result confirmed the amplification of the desired gene product.

Restriction digest, ligation, transformation and DNA sequencing

After recovery and restriction digest of the mutant genes, they were ligated into the *E. coli* expression vector pKK223-3 and were transformed into CaCl₂ competent cells of *E. coli* JM105. Colony PCR were used for pre-identification of plasmid containing positive colonies. Recombinant plasmids were isolated from the positive ones and sequenced using plasmid spe-

Table 1. Wild type PvLDH and mutated Apicomplexan LDH amino acid sequences of the amino terminal end of active site loop. PvLDH (*Plasmodium vivax* LDH), TgILDH (*Toxoplasma gondii* I LDH), TgIIILDH (*Toxoplasma gondii* II LDH), EtLDH (*Eimeria tenella* LDH) and EaLDH (*Eimeria acervulina* LDH)

| | 100 | | 108 |
|----------|---------|---|---------|
| PvLDH | F T K A | - | P G K S |
| TgILDH | L T K V | - | P G K P |
| TgIIILDH | L T K V | - | P G K S |
| EtLDH | I T K A | - | A G K S |
| EaLDH | I T K I | - | P G K S |

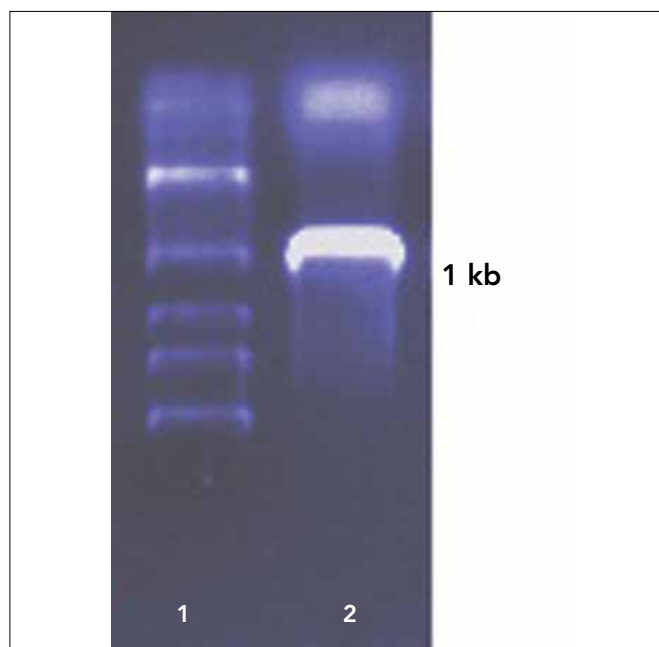


Figure 1. PCR amplicon run on EtBr stained agarose gel. 1: Marker; 2: 6xHis tagged mutant gene

cific primers. Sequencing showed presence of the addition of the 6-His tag to the C terminal of each mutant PvLDH enzymes.

Purification of the mutant PvLDH proteins

Mutant proteins expressed in *E. coli* were purified by gradient flow affinity chromatography using Ni-NTA agarose beads that have affinity to 6xHis tags of mutant proteins. According to SDS-PAGE analysis of flow through, washing and elution samples and enzyme activity measurements, purity and amount (6 mg/mL) of enzymes were found to be sufficient for kinetic analysis (Figure 2).

Western blot analysis of mutant proteins

Due to gene expression PvLDH is weak and not detected by SDS-PAGE analysis prior to purification, expression of mutant proteins were shown by Western blot analysis after the enzyme purification step. Elution 7 from every purified mutant proteins were subjected to Western blotting. Analysis results showed that mutant enzymes were at the correct size with high purity on the membrane (Figure 3).

Kinetic analysis of mutant proteins

After the affinity purification, eluted samples possessing the most pure protein samples were selected and used in the kinetic measurements. Kinetic measurements of mutant proteins were performed by using nine different pyruvate con-

centrations. 200 μ M NADH was used as cofactor. Analysis was performed in a total of 1 mL kinetic buffer containing 50mM trizma base and 50mM KCl. K_m and V_{max} values of mutant enzymes were identified by Grafit 3.0. The k_{cat} value was calculated by using these results and the amount of the enzyme used in the reaction. The resultant parameters are presented in Table 2.

Homology modelling

Mutant protein sequences were obtained by exchanging the desired aminoacids on the FASTA sequence of the wild type enzyme prior to the modelling study. Homology modelling of mutant types of the wild type enzyme were done using SWISS-MODEL, automated protein homology-modeling server. PDB files were also created by the SWISS-MODEL for each mutant protein. According to the Molsoft ICM-Pro output datas, amino acid substitutions did not completely change the conformation of the amino terminal end of the active site loop (Figure 4). Superimposition of the wild-type and mutant sequences showed that the amino terminal of the enzymes do not cause deflexion by means of carbon backbone at all. However new residues of the amino terminal end may be able to trigger some new connections with their microenvironments.

Discussion

When comparing LDH enzymes from *Plasmodiums* and other Apicomplexan parasites, some amino acids are conserved between 100th and 110th residues where the active site is located (23). However the amino acids (100F, 101T, 102K, 103A, 105P, 106G, 107K, 108S) located in the terminal end of the active site loop region shows some degree of differences among *Plasmodiums* and some other Apicomplexans (*Toxoplasma gondii* LDH1 and LDH2, *Eimeria tenella*, *Eimeria acervulina*, *Theileria parva*) (17). In this study, kinetic analyses were conducted on previously mutated *Plasmodium vivax* LDH enzymes, that were obtained by mimicking *Toxoplasma gondii* I and II, *Eimeria acervulina* and *Eimeria tenella* LDH's, to show the importance of N-terminal amino acids of the active site.

Previously mutated enzymes were produced as recombinant in *E. coli* hosts and affinity purification were achieved

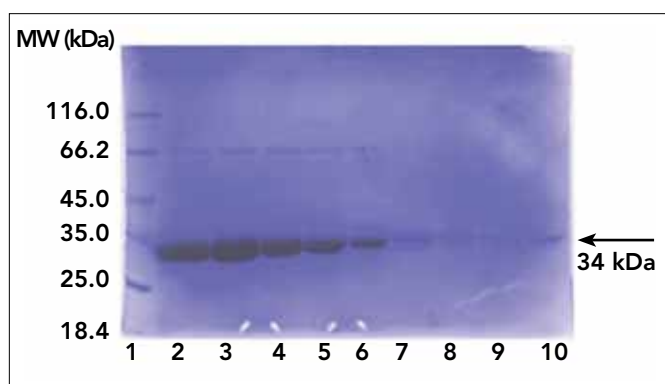


Figure 2. SDS-PAGE analysis of purified mutant protein. 1: Marker; 2-10: Elutions

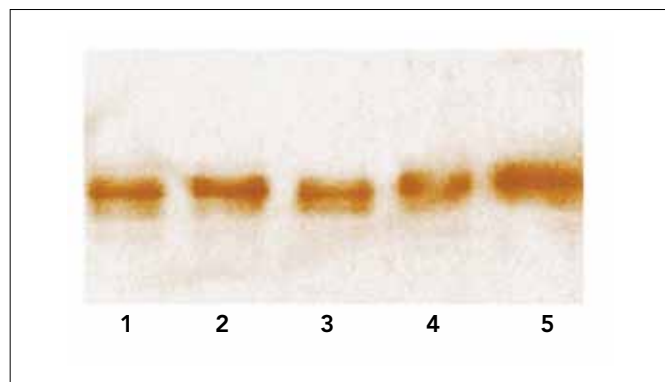


Figure 3. Western blot analysis of all mutant proteins. 1: PvLM3Tg1; 2: PvLM2Tg2; 3: PvLM3Et; 4: PvLM2Ea; 5: Wild type *P. vivax* LDH

Table 2. Steady-state kinetic data for mutant enzymes. PvLDH (wild type *Plasmodium vivax* LDH), PvLM3Tg1 (*Toxoplasma gondii* I LDH), PvLM2Tg2 (*Toxoplasma gondii* II LDH), PvLM3Et (*Eimeria tenella* LDH) and PvLM2Ea (*Eimeria acervulina* LDH)

| | K_m , pyruvate (mM) | k_{cat} , pyruvate (s ⁻¹) | V_{max} | Standart Errors, | V_{max} |
|----------|-----------------------------|---|-----------|---------------------|-----------|
| PvLDH | 0.0378 | 31.7 | 0.1413 | 0.0038 | (24) |
| PvLM3Tg1 | 0.1294 | 30.6 | 0.1506 | 0.0047 | |
| PvLM2Tg2 | 0.0811 | 29.8 | 0.1671 | 0.0034 | |
| PvLM3Et | 0.1665 | 38.15 | 0.1420 | 0.0049 | |
| PvLM2Ea | 0.1865 | 40.64 | 0.1653 | 0.0044 | |

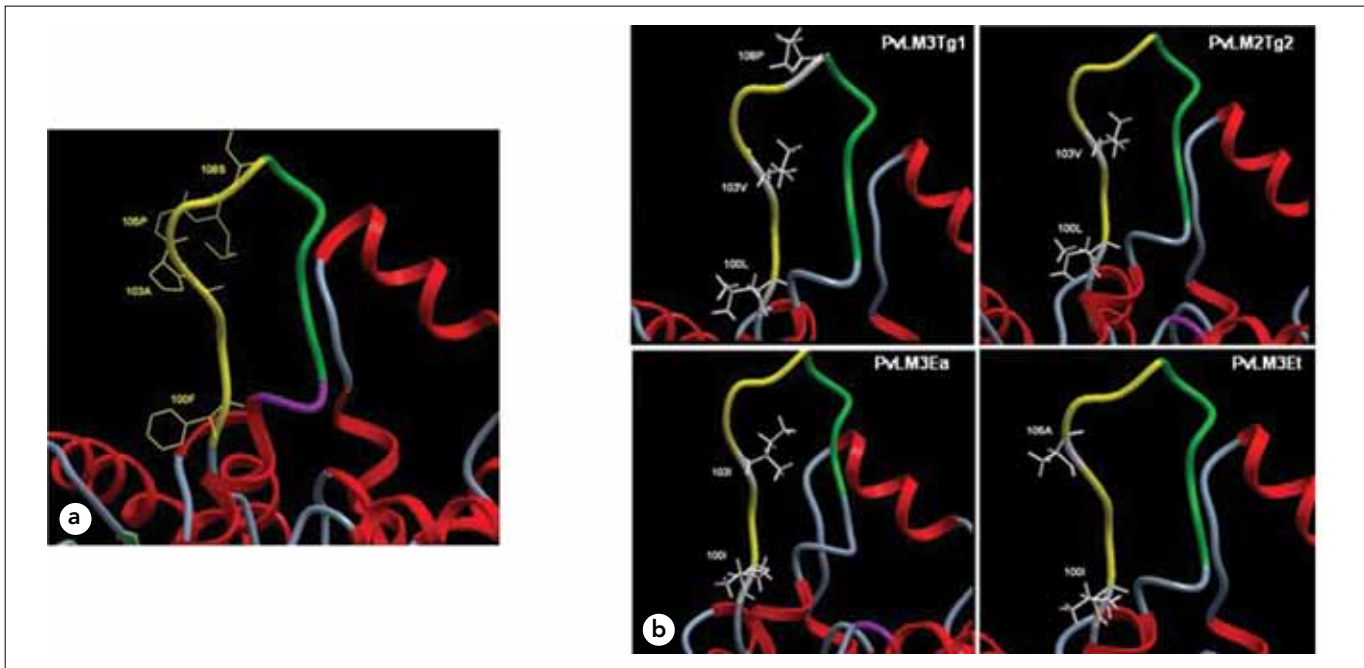


Figure 4. a) Presentation of the active site of wild type PvLDH enzyme. Yellow; amino terminal end of active site loop (residues 100F, 103A, 105P and 108S are located at the amino terminal end of active site loop), green; The five residue insertion in the active site loop. b) Showing up every four different mutations at the amino terminal end of active site loop. (yellow; amino terminal end of active site loop, green; the five residue insertion in the active site loop of the PvLDH, mutated residues are shown in white)

before kinetic analysis (17). Results of SDS-PAGE analysis indicated that the produced mutant LDH enzyme was about 34 kDa and the amount of elutions were enough for a further kinetic analysis. Western blot of all LDH enzymes proved the cloned and purified enzymes belonging to *Plasmodium* species (13). According to results taken from SDS-PAGE analysis and spectrophometric measurements, only the last 4 elutions had appropriate purity for kinetic studies.

K_m and k_{cat} values of the mutant enzymes obtained in this study were compared with the values of the wild type *P. vivax* LDH enzyme (23, 24). K_m pyruvate value of PvLM3Tg1 mutant increased to 0.1294 mM and k_{cat} value was same as the wild type enzyme. Increase of K_m value shows decreasing substrate binding affinity of the enzyme considerably while doing catalysis function normally. The K_m value of PvLM2Tg2 mutant increased about two folds compared to the wild type. On the other hand, the k_{cat} value showed no distinctive change. Kinetic studies of PvLM2Ea mutant enzyme showed a dramatic increase in K_m pyruvate value (0.1865 mM) but the k_{cat} value was not so different compared to the wild type PvLDH. Increase of K_m pyruvate value (0.1665 mM) of the PvLM3E1 mutant was much higher than the same value of TgLDHs. However the k_{cat} value was slightly higher than the other TgLDH mutant enzymes.

Comparison of the crystal structure of the *Plasmodium* LDH enzymes showed highly conserved structural models (10, 23). There were no significant alterations within the active site and cofactor binding regions (10, 23). Homology modelling of mutated sites also showed highly similar 3D structure among four enzymes (*Toxoplasma gondii* LDH1 and LDH2, *Eimeria tenella* LDH, *Eimeria acervulina* LDH). Results of superimposition be-

tween wild and mutant PvLDHs indicated unchanged carbon backbone coordinates. However, mutated residues showed some micro changes on the amino terminal end of the active site loop backbone. This could cause some new connections with their microenvironments such as water molecules. This could explain the changes in the K_m pyruvate and k_{cat} values of mutant enzymes compared to the wild type enzyme.

Conclusion

The result of mimicking the active site terminal aminoacids of *P. vivax* LDH to other *Apicomplexan* LDHs shows the importance of this site as a drug target candidate. According to kinetic studies K_m values of all mutant enzymes show some distinctive changes compared to the wild type LDH enzyme. Aminoacid changes in this region clearly cause decreasing enzyme affinity to its substrate. However k_{cat} values of all mutant proteins are nearly the same and this shows that catalytic activity of enzymes were not disrupted completely. Sensitivity of the studied region emphasizes the significance of this site for drug design studies for both *Plasmodium* and some other *Apicomplexans*.

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Conflict of Interest

No conflict of interest was declared by the authors.

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