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# Functional analysis of human thromboxane synthase polymorphic variants

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## Abstract

**Background—**Thromboxane A synthase (TXAS) metabolizes the cyclooxygenase product prostaglandin (PG) $H_2$ into thromboxane $A_2$ ($TXA_2$), a potent inducer of blood vessel constriction and platelet aggregation. Non-synonymous polymorphisms in the *TBXAS* gene have the potential to alter TXAS activity and affect $TXA_2$ generation.

**Objectives—**Assess the functional effects of genetic variants in the TXAS protein, including K258E, L357V, Q417E, E450K and T451N.

**Methods—**Wild-type TXAS and the variant proteins were expressed in a bacterial system and purified by affinity and hydroxyapatite chromatography. The two characteristic catalytic activities of TXAS were assayed in each of the purified recombinant proteins: isomerization of $PGH_2$ to $TXA_2$ and fragmentation of $PGH_2$ to 12-hydroxyheptadecatrienoic acid and malondialdehyde.

**Results—**All of the variants exhibited both isomerization and fragmentation activities. The $K_M$ values of the variants ranged from 27–52 μM $PGH_2$ (wild-type value: 32 μM $PGH_2$); $V_{max}$ values of the variants ranged from 18–40 units/mg (wild-type value: 41 units/mg). The kinetic differences were largest for the L357V variant, whose $V_{max}$ / $K_M$ ratio was just 27% of the wild-type value.

**Conclusion—**The increased $K_M$ and decreased $V_{max}$ values observed with L357V suggest this variant may generate less $TXA_2$ at the low levels of $PGH_2$ expected *in vivo*, raising the possibility of attenuated signaling via the thromboxane pathway.

### Keywords

cytochrome p450; thromboxane synthase; genetic polymorphism; enzyme kinetics

## INTRODUCTION

Thromboxane $A_2$ ($TXA_2$) is a potent stimulator of platelet secretion and aggregation as well as a vasoconstrictor with an important role in major cardiovascular diseases, such as atherosclerosis and myocardial infarction [1,2,3]. $TXA_2$ is biosynthesized by the sequential actions of prostaglandin H synthase (also known as cyclooxygenase), which converts arachidonic acid to prostaglandin $H_2$ ($PGH_2$), and thromboxane synthase (TXAS) which subsequently converts $PGH_2$ to $TXA_2$ (Fig. 1). TXAS is a cytochrome P450 enzyme (also

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known as CYP5A1[4]) that is associated with the endoplasmic reticulum membrane and is primarily expressed in hematopoietic cells, such as platelets, monocytes, leukocytes and macrophages [5]. Unlike other microsomal P450s, which catalyze a mono-oxygenation reaction and require P450 reductase as electron donor, TXAS catalyzes an isomerizaton reaction without an external electron donor or molecular oxygen [6]. Notably, besides forming $TXA_2$, TXAS fragments $PGH_2$ to 12-hydroxy-5,8,10-heptadecatrienoic acid (12-HHT) and malondialdehyde (MDA) in a ratio of 1:1:1 [6] (Fig. 1). The biological functions of MDA and HHT are unclear, although MDA can form adducts with amino groups of proteins or phospholipids and such adducts have been detected in atherosclerotic lesions of human aorta [7]. MDA also participates in formation of an important endogenous DNA adduct that may contribute to human genetic disease and cancer [8,9].

TXAS is a single copy gene, located on chromosome 7q33-35 [10]; the gene is 193 kb long and has 13 exons [11]. Knockout of the corresponding mouse gene resulted in mild bleeding disorders and altered vascular responses to arachidonic acid [12], and knockdown of the zebrafish gene for TXAS resulted in defective heart development [13]. Further, human TXAS mutations have been linked to a bone density disorder [14]. Fourteen non-synonymous polymorphisms in the TXAS coding region were identified in the first three reports on variations in the gene [15,16,17], with evidence for selective evolutionary pressure against genetic mutations in the gene [17]. In view of the roles of TXAS in physiology and pathology, it is important to understand the effects of human TXAS protein variants on catalytic activity. We have developed a prokaryotic expression system that provides sufficient recombinant TXAS with ~90 % purity for enzymatic characterization [18,19]. Structural analysis based on homology modeling suggested that four of the TXAS variants (K258E, Q417E, E450K and T451N) were likely to have altered enzymatic activities [17]. In addition, the L357V variant, which has a minor allele frequency of almost 11% in African-Americans [17] was predicted to have altered catalytic activity by the PolyPhen algorithm [20]. The present report describes the results of our enzymatic characterization of the five targeted TXAS variants as purified recombinant proteins.

## MATERIALS AND METHODS

### Materials

Arachidonic acid was from NuChek Preps (Elysian, MN). IPTG, δ-aminolevulinic acid, Igepal CA630 and Lubrol PX were acquired from Sigma. Nickel nitrilotriacetate agarose and hydroxyapatite were obtained from Qiagen and Bio-Rad, respectively. $PGH_2$ was synthesized from arachidonic acid using detergent-solubilized ovine prostaglandin H synthase-1 and was purified by normal phase chromatography [21].

### Protein expression and purification

The recombinant TXAS proteins were prepared following procedures described previously [18,19]. Briefly, the wild-type TXAS cDNA was modified by replacement of the first 28 amino acid codons with a sequence coding for a hydrophilic segment (MAKKTSS) and by addition of four histidine codons for a his-tag at the carboxyl terminus. The cDNA was subcloned into the pCW expression plasmid to produce the wild-type vector, pCW-TXAS.

Site-directed mutagenesis to produce expression vectors for the five variants was performed using the Quick-Change kit (Stratagene, La Jolla, CA, USA) with pCW-TXAS as the template. The mutations were confirmed by DNA sequencing. Each construct was transformed into *E. coli* BL21(DE3)pLys and expression of recombinant protein was induced by IPTG in the presence of the heme precursor, δ-aminolevulinic acid. Each recombinant TXAS protein was solubilized with 2% Igepal CA630 and was purified by sequential chromatography on nickel affinity resin and hydroxyapatite, and stored at −70°C. The concentration of each purified recombinant TXAS was determined using a Soret extinction coefficient of 100 $mM^{-1}$ $cm^{-1}$ [18]. Protein concentrations were determined using a Bio-Rad Protein Assay Kit with bovine serum albumin as standard.

### TXAS enzymatic assays

To determine the $TXA_2$-synthesizing activity, TXAS (30 nM) in 200 μl of 20 mM $NaP_i$, pH 7.4, and 0.2 % Lubrol PX at room temperature (23 °C) was reacted with the desired level of $PGH_2$. The reaction was terminated after 15 s by acidification with 21 μl of 2 M citric acid. Formation of $TXB_2$, the stable hydrolysis product of $TXA_2$, was measured by radioimmunoassay [22]. To monitor continuously the initial rate for steady-state kinetic analysis, the assay was carried out at room temperature (23 °C) in a cuvette containing 400 μl of 20 mM $NaP_i$, pH 7.4, 0.2 % Lubrol PX and 5 nM of recombinant TXAS. The reaction was started by an addition of the desired level of $PGH_2$ and the $A_{268}$ monitored for the first 30 s. The initial rate was calculated from the increase in $A_{268}$ resulting from formation of MDA ($\varepsilon = 31.5\ mM^{-1}\ cm^{-1}$). One unit is the amount of TXAS forming 1 μmol of MDA per min. The TXAS rate data for a series of $PGH_2$ concentrations were fitted to the Michaelis-Menten equation by non-linear regression to estimate values for $K_m$ and $V_{max}$. Each assay was performed in triplicate.

### Statistical analysis

The p-values were generated using the *t*-test to determine any significant differences in kinetic parameters between the means of wild-type and mutants.

## RESULTS AND DISCUSSION

### Production of variant TXAS proteins

The expression levels of the K258E, L357V and E450K variants were similar to that of a wild-type TXAS (~0.27 μmol/liter of culture), whereas the Q417E and T451N variants were expressed at lower levels (0.07–0.09 μmol/liter of culture). Note that differences in expression level among TXAS variants in the present bacterial system are unlikely to reliably predict the expression levels of the variants in native human tissues. Each of the recombinant proteins was purified to ~90% homogeneity, as judged by SDS-PAGE analysis (Fig. 2). The optical absorbance spectrum of resting wild-type TXAS had a Soret peak at 418 nm, as previously observed [18]. Each of the TXAS variants also exhibited a Soret peak near 418 nm, except for K258E, whose Soret peak was at 422 nm (data not shown). These results indicate that the heme in the wild-type and each of the TXAS variants is predominantly in the expected six-coordinate, low-spin ferric state.

The initial enzymatic characterization of the purified TXAS variants involved measuring their ability to convert $PGH_2$ to $TXA_2$, which was quantified as its stable hydrolysis product, $TXB_2$. These screening reactions used fixed concentration of $PGH_2$ (290 μM) and enzyme (30 nM). The results, shown in Table 1, confirm that each of the variants was able to synthesize $TXA_2$ at initial rates that were 40–90% of the rate for the wild-type enzyme. Thus, none of the structural changes in the variants blocked the proper active site positioning of the $PGH_2$ substrate that is thought to be crucial for the TXAS isomerization reaction (left branch in Fig. 1) [23]. The rank order of the rate of thromboxane formation was: wild-type > T451N > Q417E > E450K > K258E > L357V (Table 1). This ranking gives a qualitative indication of the relative effects of the variants on TXAS catalysis. However, the discontinuous nature of the assay which has only one time-point, 15 s in the current assay, and possible variations in the extent of conversion of $TXA_2$ to $TXB_2$ limit quantitative interpretation of the results.

For a more detailed analysis of kinetic parameters in the variants, we monitored the second catalytic activity of TXAS, namely the fragmentation of $PGH_2$ into 12-HHT and MDA (right branch in Fig. 1). Formation of MDA gives an increase in $A_{268}$, allowing the initial, steady-state rate in each reaction to be calculated from the $\Delta A_{268}/\Delta t$ over the first 30 s. The initial rate was found to be a saturable function of the $PGH_2$ level for wild-type TXAS and each of the variants (Fig. 3). Accordingly, each set of rate vs. [$PGH_2$] data was fitted to the Michaelis-Menten equation to estimate values for $V_{max}$ and $K_M$, which are shown in Table 2. Wild-type TXAS had a $K_M$ of 32 μM $PGH_2$ and $V_{max}$ of 41 units /mg protein, values comparable to those previously reported [19,23]. The $K_M$ values of Q417E and E450K were essentially indistinguishable from the wild-type value, but those of the K258E, L357V and T451N variants were about 1.5-fold higher (Table 2), suggesting that the interaction with substrate was modestly weaker in the latter three variants. The L357V variant exhibited a marked decrease in $V_{max}$ (only 44% of the wild-type value), whereas modestly lower $V_{max}$ values were seen with K258E, E450K and T451N (81–83% of wild-type value). The $V_{max}$ was not affected in Q417E (Table 2).

The $V_{max}/K_M$ ratio, or specificity constant, is an index of catalytic rate at substrate concentrations well below the $K_M$ value [24]. Given that cellular $PGH_2$ levels are likely to be well below the $K_M$ values of all the TXAS proteins, comparison of the specificity constant values in Table 2 predicts the possible *in vivo* functional impacts of the TXAS polymorphisms. The specificity constant for Q417E is not significantly different from the wild-type value, suggesting that the variant has little functional effect (p value > 0.05). Moderate decreases in TXAS catalytic efficiency might be anticipated for the K258E, E450K and T451N variants ($V_{max}/K_M$ 50–56% of wild-type value; 0.05 > p values > 0.001). On the other hand, the $V_{max}/K_M$ value for L357V is only 27% of the wild-type value (Table 2; p value= 0.0004), predicting a large decrease in catalytic function for this variant *in vivo*.

Taken together, L357V is the most affected variant for $TXA_2$ synthesis. It should be noted that of the variants examined here, Leu357 is the only amino acid residue that is conserved in the wild-type for human, chimpanzee, dog, cow, mouse, rat, chicken and zebrafish TXAS. We have postulated previously that Leu357 is located at the end of the Helix I and suggested that a mutation at Leu357 would affect catalytic activity [17,25]. This prediction from our

computational model of the TXAS structure (Fig. 4) remains to be tested by a direct structure determination. The prevalence of L357V is an important factor in evaluating its potential impact. The dbSNP data base lists a minimal allele frequency (MAF) of 0.109 for the L357V polymorphism [26]. Thus, most individuals with this TXAS variant will be heterozygotes. Assuming that the wild type and L357V variant proteins act independently, TXAS activity in a heterozygous individual might be expected to be an average of wild type and L357V variant values. Notably, we found no evidence for linkage disequilibrium between any two polymorphisms in the study ($r^2 < 0.005$). Furthermore, these polymorphisms are relatively rare (0.6 %–11 %) and have different minor allele frequencies in different ethnic groups [17], so that any two SNPs are rarely, if ever, in combination with one another, making it very unlikely that these polymorphisms would form common haplotypes.

In general, the type and quantity of prostanoids produced from $PGH_2$ in a given cell type are governed by the specific secondary prostanoid-synthesizing enzymes present in that particular cell type [27,28]. The many variables in the prostanoid synthetic pathway in vivo make it difficult to model accurately in a cultured cell model. However, it is reasonable to expect that any altered function in a TXAS variant would change the proportion of $TXA_2$ in the prostanoid profile, with the potential to affect cellular signaling via $TXA_2$. $TXA_2$ is a critical signal in platelet stimulation, aggregation, and vasoconstriction, and increases in TXAS expression have been associated with a variety of cancers (reviewed in [29]). Thus, TXAS has become a suggested target for chemotherapy, with the development of TXAS-specific inhibitors such as ozagrel and furegrelate yielding promising early results in vitro [30,31,32]. TXAS variants with decreased activity could affect targeted drug intervention, as individuals with lower baseline TXAS activity may get less benefit from selective TXAS inhibitors. Recent findings indicate that genetic variation in TXAS is associated with several pathologies, including non-fatal myocardial infarction [33], cerebral infarction [34], and breast cancer [35]. In addition, pharmacogenetic consequences of TXAS polymorphisms on aspirin sensitivity have been investigated in stroke [36] and asthma patients [37]. Given the several pathophysiological functions of TXAS and the potential clinical utility of TXAS pathway inhibitors, it will be of interest to evaluate phenotypic and pharmacogenetic effects in individuals with the L357V polymorphism in TXAS.

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## The abbreviations used are

| | |
|---|---|
| **$TXA_2$ and $TXB_2$** | thromboxane $A_2$ and $B_2$ |
| **TXAS** | thromboxane synthase |
| **$PGH_2$** | prostaglandin $H_2$ |
| **MDA** | malondialdehyde |

| | |
|---|---|
| **HHT** | 12-hydroxy-5,8,10-heptadecatrienoic acid |

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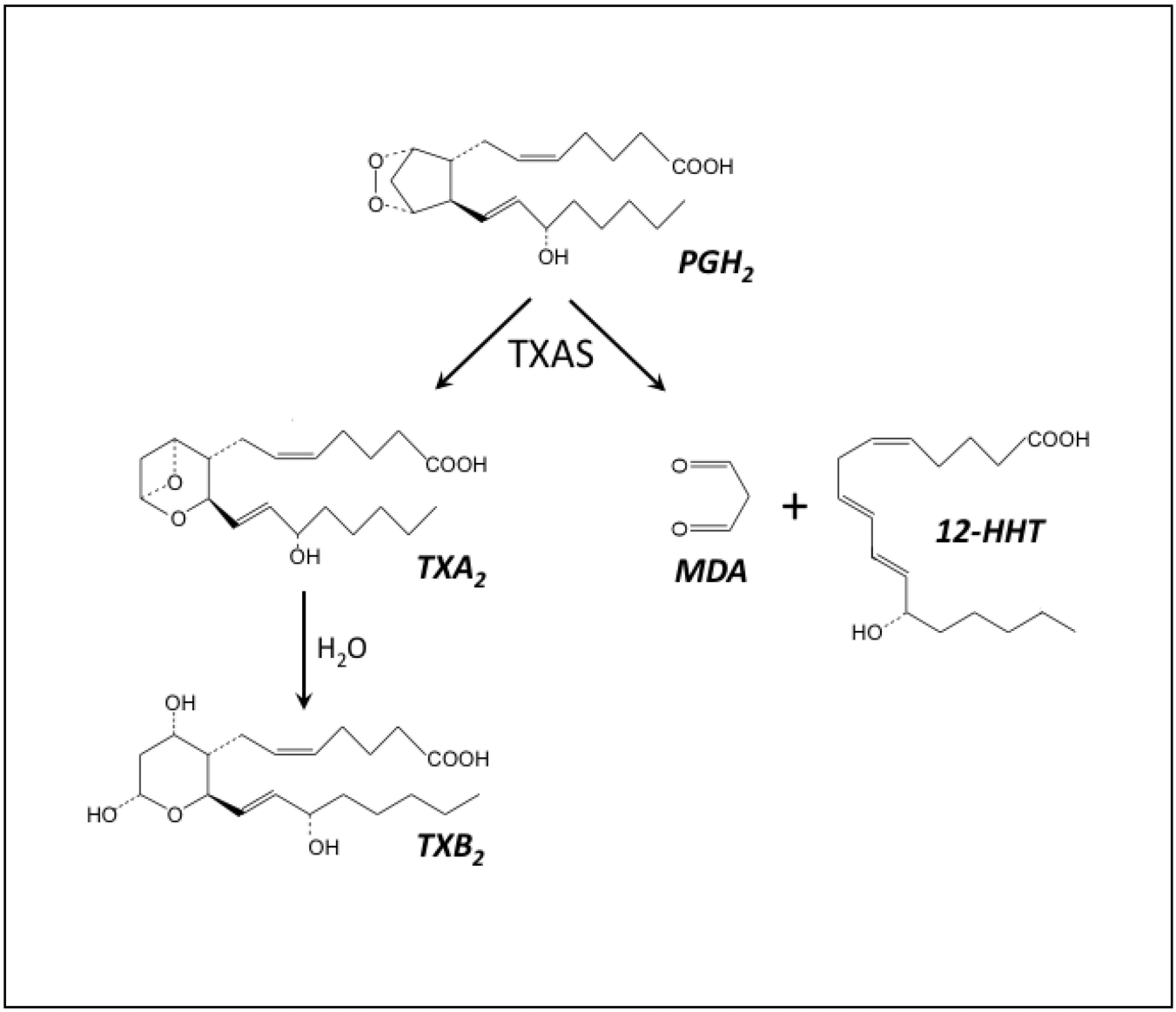


**Figure 1.**

Reactions of $PGH_2$ catalyzed by TXAS. Left branch: isomerization to $TXA_2$. Right branch: fragmentation to MDA and 12-HHT. Hydrolysis of $TXA_2$ to $TXB_2$ is nonenzymatic.

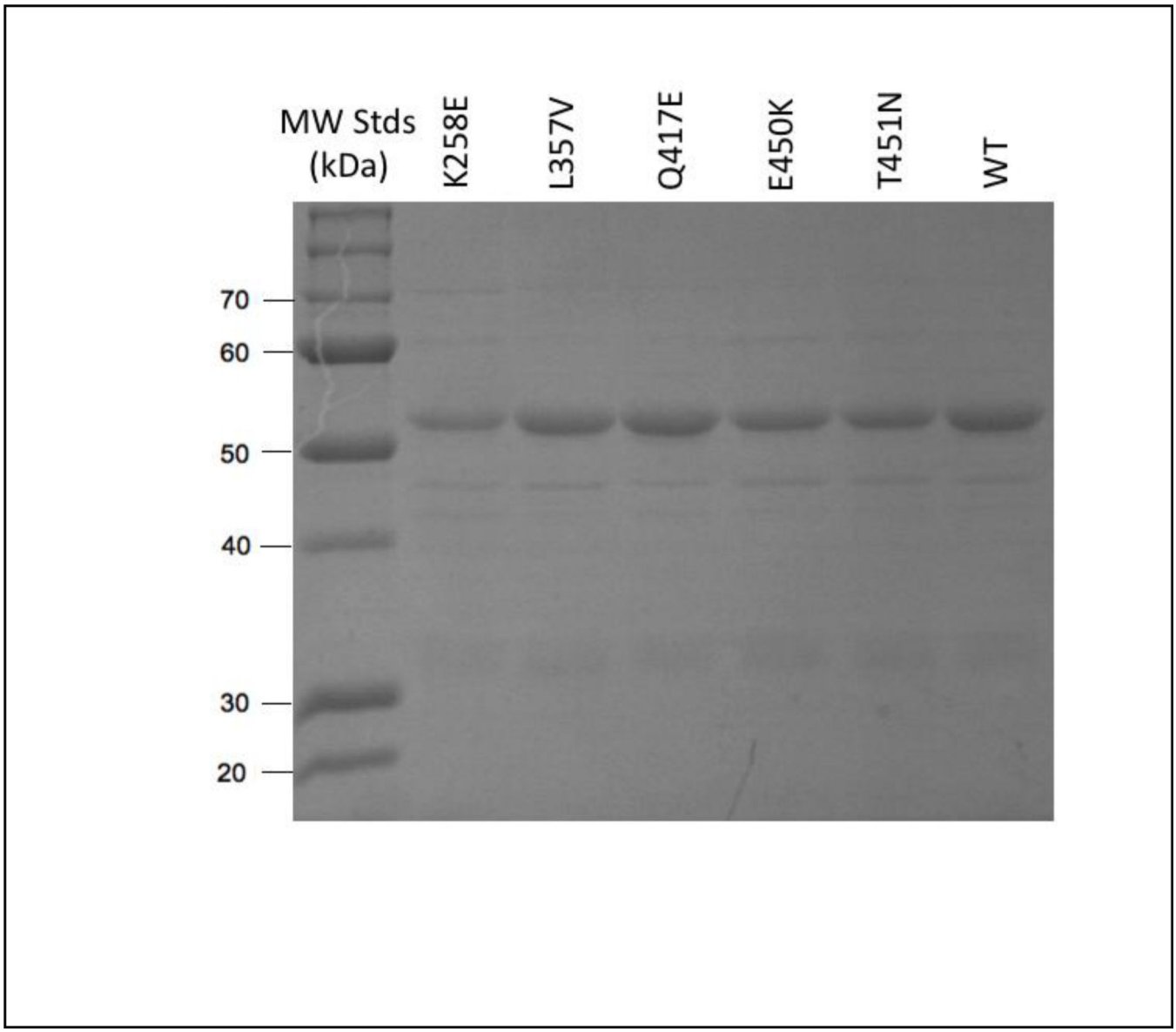


**Figure 2.**

Electrophoretic analysis of purified recombinant TXAS wild-type (WT) and variant proteins. Aliquots of purified wild-type and variant TXAS (~ one μg protein) were separated on a 10 % polyacrylamide gel and stained with Coomassie blue.

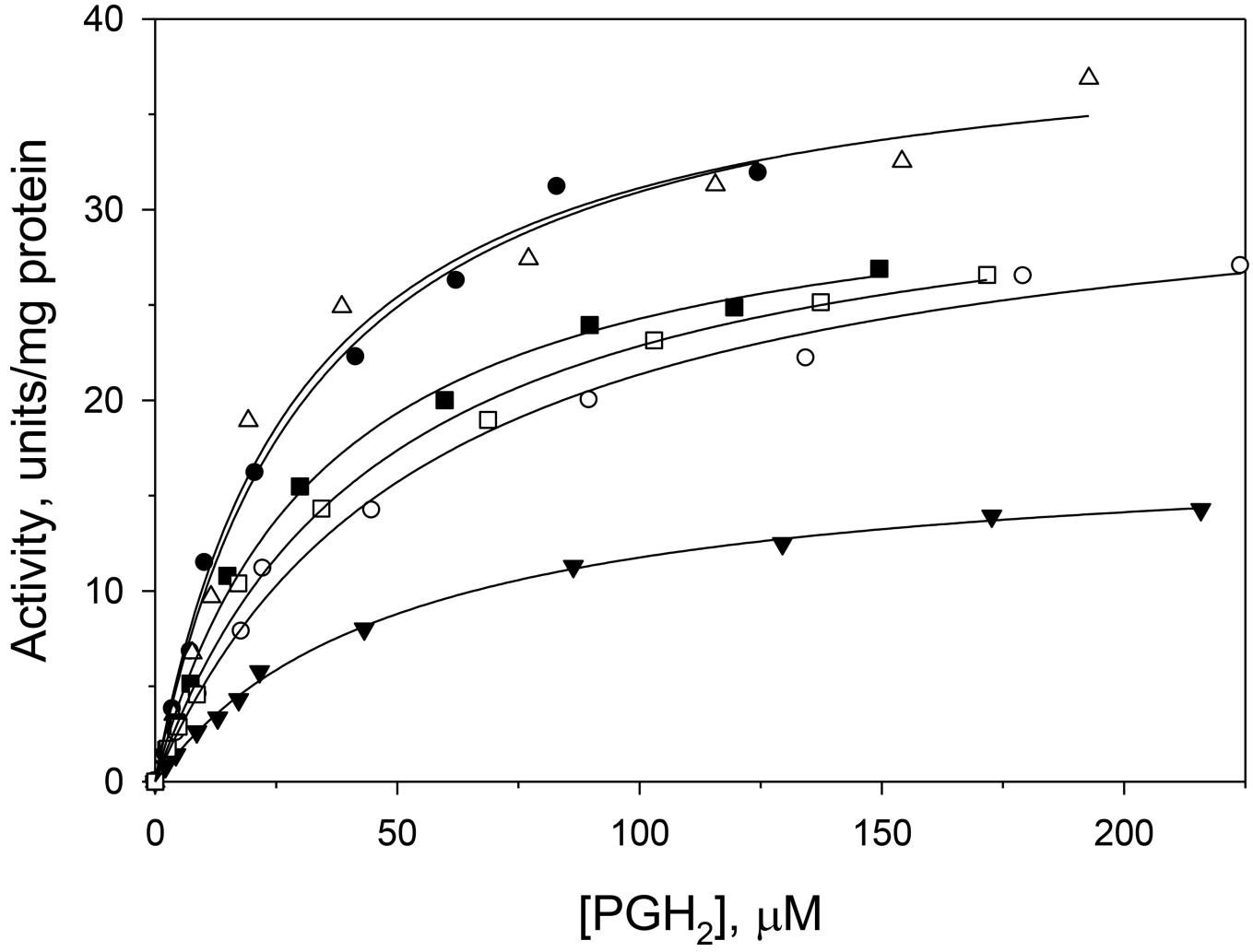


**Figure 3.**

Effect of $PGH_2$ level on reaction rate in TXAS wild-type and variants. Continuous assays contained 5 nM of recombinant TXAS. Lines represent fits of the data to the Michaelis-Menten equation. Filled circles, wild-type TXAS; open circles, K258E; filled triangles, L357V; open triangles, Q417E; filled squares, E450K, and open squares, T451N. Data shown is from a representative set of experiments

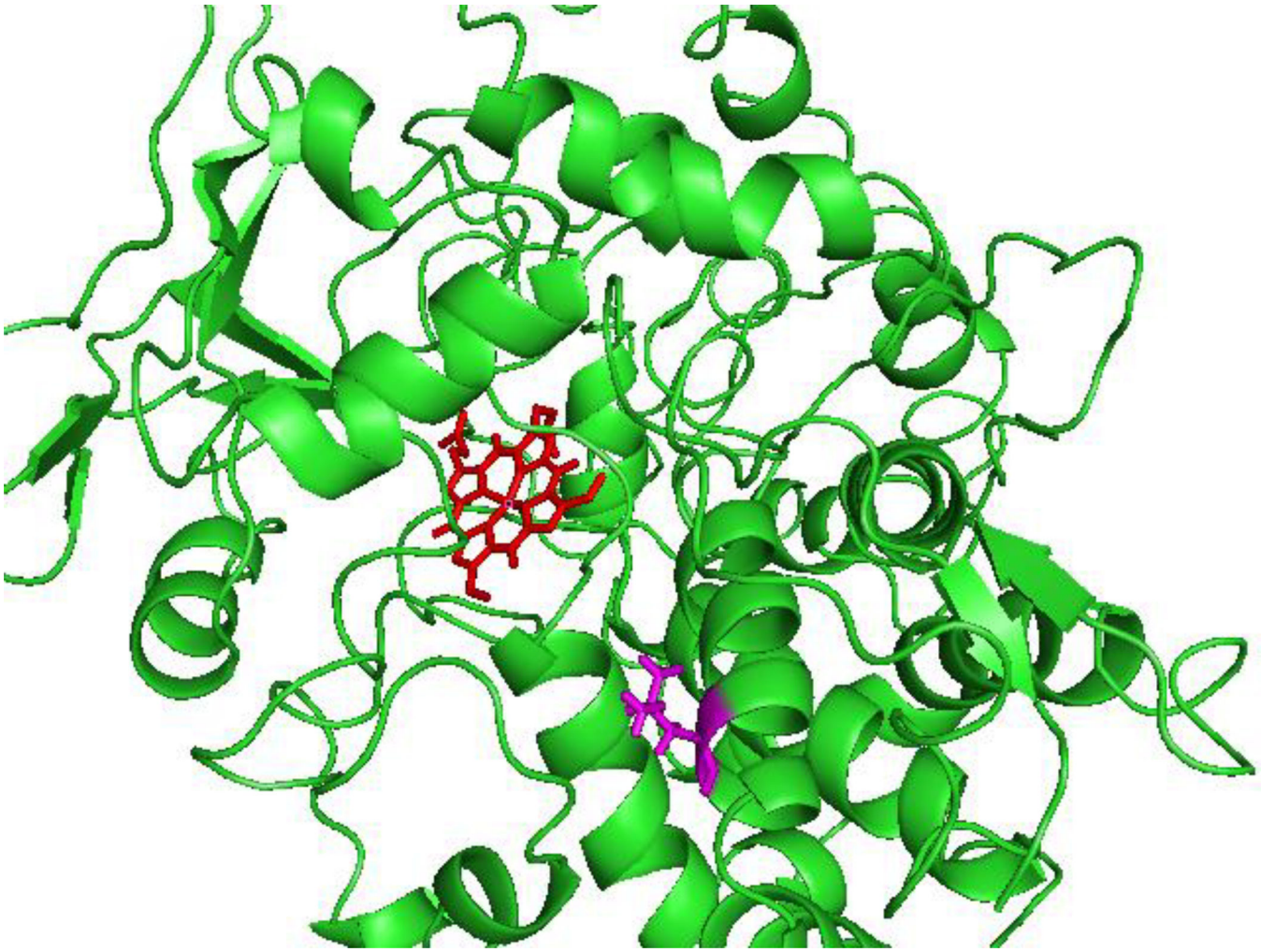

**Figure 4.**
Computation model of TXAS structure [38] showing the position of Leu357 (*magenta*) and heme (*red*). The figure was generated using PyMol (The PyMol Molecular Graphic System, Version 1.3, Schrödinger, LLC).

## Table 1

$TXB_2$ synthesis by purified recombinant wild-type and variant TXAS proteins.

| TXAS construct | Specific activity (μmol $TXB_2$/min/mg)[a] |
|---|---|
| wild-type | 9.9 ± 0.5 |
| K258E | 5.7 ± 0.1 |
| L357V | 4.2 ± 0.1 |
| Q417E | 7.1 ± 0.4 |
| E450K | 6.3 ± 1.2 |
| T451N | 8.8 ± 0.9 |

[a] In reactions with 290 μM $PGH_2$ and 30 nM enzyme. Mean ± SEM are shown for three measurements.

**Table 2**

TXAS kinetic parameters for purified wild-type and variant proteins[a].

| | wild-type | K258E | L357V | Q417E | E450K | T451N |
|---|---|---|---|---|---|---|
| $K_M$, μM $PGH_2$ | 32.0 ± 2.7 | 51.9 ± 4.3 (p= 0.017)[b] | 51.0 ± 2.5 (p= 0.007) | 27.1 ± 3.6 (p> 0.05) | 33.8 ± 2.4 (p> 0.05) | 46.7 ± 3.9 (p= 0.036) |
| $V_{max}$, units /mg protein | 40.8 ± 1.0 | 32.9 ± 1.0 (p= 0.005) | 18.1 ± 1.0 (p= 0.0001) | 39.7 ± 1.0 (p> 0.05) | 32.9 ± 1.0 (p= 0.005) | 34.0 ± 1.0 (p= 0.009) |
| $V_{max}$ /$K_M$, units/mg/μM $PGH_2$ | 1.28 ± 0.14 | 0.64 ± 0.07 (p= 0.015) | 0.35 ± 0.04 (p=0.0031) | 1.46 ± 0.23 (p> 0.05) | 0.97 ± 0.10 (p> 0.05) | 0.72 ± 0.08 (p= 0.025) |

[a] Activity was assayed by following the absorbance changes at 268 nm for MDA formation in the presence of 5 nM TXAS. Values represent mean ± SEM of three experiments.

[b] p-values are calculated as compared to the wild-type, and values larger than 0.05 are considered to be statistically in-significant.