Identification of Thromboxane A\textsubscript{2} Synthase Active Site Residues by Molecular Modeling-guided Site-directed Mutagenesis\textsuperscript{*}

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Human thromboxane A\textsubscript{2} synthase (TXAS) exhibits spectral characteristics of cytochrome P\textsubscript{450} but lacks monooxygenase activity. Its distinctive amino acid sequence makes TXAS the sole member of family 5 in the P\textsubscript{450} superfamily. To better understand the structure-function relationship of this unusual P\textsubscript{450}, we have recently constructed a three-dimensional model for TXAS using P\textsubscript{450}\textsubscript{BM-3} as the template (Ruan, K.-H., Milfeld, K., Kulmacz, R. J. , and Wu, K. K. (1994) Protein Eng. 7, 1345–1551) and have identified a potential active site region. The catalytic roles of several putative active site residues were evaluated using selectively mutated recombinant TXAS expressed in COS-1 cells. Mutation of Ala-408 to Glu or Arg-413 to Gly led to a complete loss of enzyme activity despite expression of mutant protein levels equivalent to that of the wild-type TXAS. Mutation of Ala-408 to Glu or Leu retained the enzyme activity at levels of 30 or 40\%, respectively. This suggests that Ala-408 provides a hydrophobic environment for substrate binding. Mutation of Arg-413 to Lys or Gln completely abolished the enzyme activity, indicating that this residue is essential to catalytic activity and supports its identification as an active site residue. Mutation of Arg-410 to Gly or Glu-433 to Ala resulted in >50\% reduction in the enzyme activity without appreciably altering mutant protein expression, consistent with a more subtle effect of these residues on TXAS catalytic efficiency. Mutation of residues predicted to be involved in binding the heme prosthetic group, including the heme thiolate ligand Cys-480, Arg-478, Phe-127, and Asn-110, each markedly reduced the expressed protein level and abolished enzyme activity. This suggests that proper heme binding is important to synthesis or stability of recombinant TXAS. Mutation of Ile-346, which corresponds to P\textsubscript{450}\textsubscript{BM-3}-Thr-252, an essential amino acid involved in dioxygen bond scission, to Thr increased the enzymatic activity by 40\%, suggesting that oxygen bond cleavage is not a rate-limiting step in thromboxane A\textsubscript{2} biosynthesis. The present results from site-directed mutagenesis support the overall structure of the TXAS active site predicted by homology modeling and have allowed refinement of the position of bound substrate.

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The abbreviations used are: TXA\textsubscript{2}, thromboxane A\textsubscript{2}; TXB\textsubscript{2}, thromboxane B\textsubscript{2}; TXAS, thromboxane A\textsubscript{2} synthase; PGH\textsubscript{2}, prostaglandin H\textsubscript{2}; kb, kilobase; U, U6619, 15(5)-hydroxy-11,19-(epoxymethano)prosta-5,13(Z)-diene acid; P450\textsubscript{BM-3}, cytochrome P450 camphor (CYP101); P450\textsubscript{BM-3}, cytochrome P450 Baecillus megaterium-3 (CYP102); P450\textsubscript{BM-3}, cytochrome P450 terpinel (CYP108).
atations of several amino acid residues expected to be in the heme environment, including the conserved thiolate ligand Cys-480, caused a marked reduction in the expressed protein levels, suggesting that proper heme binding is required for normal TXAS synthesis or stability.

EXPERIMENTAL PROCEDURES

Materials—COS-1 cells (ATCC CRL-1650) were obtained from the American Type Culture Collection. The eukaryotic expression vector pSG5 was from Stratagene. Cell culture media and antibiotics were from Life Technologies, Inc. Oligonucleotides were synthesized by Genosys (The Woodlands, TX). Leupeptin, pepstatin, aprotinin, and Pefabloc SC were obtained from Boehringer Mannheim. DEAE-dextran was from Sigma. [1-14C]Arachidonic acid was from Amersham Corp. PGH$_2$ and 15-deoxy-Delta-12,14-prostaglandin J$_2$ (15-dPGJ$_2$) were from Cayman Chemical (Ann Arbor, MI).

Molecular Modeling of Three-dimensional Structure—A detailed working model of the TXAS three-dimensional structure has been constructed using a homology-modeling technique (12). Briefly, a sequence similarity alignment was made for TXAS and the homoprotein domain of P450cam by incorporating secondary structural predictions and experimental information, including conserved residues and heme ligand identity. The final alignment shows an overall 26.4% residue identity and 48.4% residue similarity between these two proteins. The main chain conformation of TXAS was then built with a Quanta 3.3 protein-conformation search. PGH$_2$ was initially docked into the proposed TXAS substrate binding site, which corresponds to the P450cam substrate binding site. Finally, an energy minimization routine with 500 steps of steepest descent was performed for the TXAS model containing heme and PGH$_2$ structures to remove steric clashes between atoms and to produce reasonable protein folding. The same approaches were used to modify the three-dimensional structure of the TXAS-PGH$_2$ complex based on the site-directed mutagenesis study in this article.

Site-directed Mutagenesis—A vector coding for TXAS was constructed by inserting the TXAS cDNA at the EcoRI site of the eukaryotic expression vector pSG5, which uses the SV40 early promoter to facilitate in vivo expression (7), to obtain pSG5-TXAS. The construct was validated to have a correct orientation with respect to the SV40 promoter in pSG5 by appropriate restriction enzyme digestion. Ural containing single-stranded pSG5-TXAS was used to introduce specific base changes into TXAS DNA. Single-stranded oligonucleotides containing the base mismatch(es) was incubated with the single-stranded DNA at 70°C, and then the mixture was allowed to cool to 30°C over 30 min. The oligonucleotide was extended and ligated by Sequenase and T4 DNA ligase. The resultant duplex DNA was used to transform E. coli XL-1 Blue cells. Plasmids with the desired DNA mutations were verified by double-stranded sequencing using a Sequenase kit (U. S. Biochemical Corp.) or a double-stranded cycle sequencing kit (Life Technologies, Inc.). The oligonucleotides used for the mutations were (in 5’ to 3’ direction with mutated bases underlined): Cys-480→Ser, GAGCTCTCAGGCTGCTG; Arg-413→Gly, AGATTCACAGGGAGCCAG; Arg-478→Ala, GGCCAGCGACTGCTG; Thr-505→Ser, ACATCACGACTTACGCTG; Glu-433→Glu, CACAGCTTCTGTCCAGAC; Gly-408→Ala, GCCAGACCAAGCGCAGC; and Ile-346→Ser, GCCAGACCGTGCTG; Thr-350→Thr, TCAAGAATTTTCCGACG; Arg-410→Gly, GAAACCTGGCGGGTAC. Polymerase chain reaction was carried out to generate a 0.4-kb fragment by amplifying the wild-type TXAS cDNA with the sense primer and an antisense primer corresponding to nucleotides 1601–1621 downstream from the translational start codon. In a separate tube, a 0.15-kb fragment was generated by polymerase chain reaction using the antisense primer and a sense primer corresponding to nucleotides 1062–1081 downstream from the translation start codon. Both 0.4- and 0.15-kb fragments were combined, diluted 200-fold, and used as templates for polymerase chain reaction in a buffer containing 100 mM each of the primers 1601–1621 and 1062–1081 plus 1 mM each of the sense and antisense primers. The resultant 0.55-kb fragment was digested with HindIII and KpnI to obtain a 0.43-kb DNA. The 0.43-kb fragment was gel purified and subcloned into the unique HindIII-KpnI sites of pSG5-TXAS. Mutants Ala-408→Leu, Arg-413→Lys and Arg-413→Gln were generated identically, except that the primers used for mutations were Ala-408→Leu, AGCCGCACTTTGATTCAGT; and ATCTGAAATTTTCCGACG; and Arg-413→Gln, AGATTCACAGGGAGCCAGC and CTGCTCAGTGATCCGTGCTG. The mutants were confirmed by DNA sequencing. The plasmids for transfection were prepared using Qiagen plasmid purification kits (Qiagen, Inc., Studio City, CA).

Transfection of COS-1 Cells—COS-1 cells were grown to near confluency in 100-mm tissue culture dishes at 37°C in a humidified 5% CO$_2$ atmosphere with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Transfection was carried out using 10–100 μg of DNA using calcium phosphate precipitation. The transfected cells were further incubated for 40 h until harvest.

Preparation of TXAS Cell Microsomes—Cells were scraped from the plates, pelleted by centrifugation at 900×g for 10 min, washed with phosphate-buffered saline, and sonicated by a Sonifier Cell Disruption, model W185 (five 10-s pulses) in 5 ml of phosphate-buffered saline containing 1 μM aprotinin, 1 mM EDTA, 10 mM pepstatin, 10 μg/ml leupeptin, 0.5 mM Pefabloc SC, and 0.1% Triton X-100. After removing cell debris by centrifugation at 10,000×g for 10 min, a microsomal fraction was isolated by centrifugation at 105,000×g for 1 h, and then resuspended in 400 μl of phosphate-buffered saline. Protein concentrations were determined by biuretichronic acid assay using bovine serum albumin as a standard.

Determination of TXAS Protein Level by Western Blot—Immunoblotting was performed according to the procedure previously described (9, 23). In brief, microsomal samples were boiled in electrophoresis sample buffer (24) for 4 min before applying to a 10% polyacrylamide minigel for electrophoresis. The resolved proteins were transferred electrophoretically at 200 mA for 1 h to a nitrocellulose membrane. Subsequently, the membrane was blocked with 5% nonfat milk in phosphate-buffered saline and probed with a 1:500 dilution of rabbit antibody against TXAS conjugated to glutathione S-transferase (9) at 4°C overnight, followed by incubation with 10 μg/ml goat anti-rabbit IgG conjugated to horseradish peroxidase, and finally visualized by incubation with the peroxidase substrate 4-chloro-1-naphthol (Sigma).

Activity Assays—TXAS activity was assayed by incubating 0.5 μM PGH$_2$ for 2 min at 37°C in the absence or presence of 150 μM U46619, a TXAS inhibitor. The reaction was stopped by acidification, and TXB$_2$ was measured by radioimmunoassay as described previously (25). TXAS activity values are reported as the amount of TXB$_2$ produced per μg of total protein per min.

To determine K$_{n}$ and V$_{max}$ values, microsomal proteins (50 μg) from COS-1 cells transfected with wild-type or mutant TXAS DNA were incubated with 0–10 μM PGH$_2$ for 2 min at 37°C in the absence or presence of 150 μM U46619, a TXAS inhibitor. The reaction was stopped by acidification, and TXB$_2$ was measured by radioimmunoassay. Estimations of the kinetic parameters were obtained from nonlinear least squares fits to the Michaelis-Menten equation, using the Marquardt-Levenberg algorithm.

RESULTS AND DISCUSSION

Predicted Substrate Binding Region—Based on the sequence alignment and crystal structure of P450cam, Gotoh and Fujii-Kuriyama (26) predicted six substrate binding domains in P450. One of these domains is located between helix K and the conserved heme binding Cys region (Fig 1). In this region, a β sheet configuration was identified, and two pairs of antiparallel β strands (Fig 1, p-β7 and β8–p-7) were conserved in the crystal structures of P450cam, P450BM-3, and P450cyp2c. The p-β8 strand was considered part of the substrate binding sites for these P450s (13, 27, 28). Ravichandran et al. (13) predicted...
that, in this region of P450BM-3, three amino acid residues (Ala-328, Ala-330, and Met-354) were involved in the substrate binding. Our three-dimensional model of TXAS (12) also suggested that this β sheet region located between Pro-407 and Val-436 was involved in substrate binding, with the corresponding amino acid residues being Ala-408, Arg-410, and Glu-433. These residues are indicated by lines below the sequences. A, α helices; β, β-sheets; β strand. +, amino acid residues studied in this work. Numbers of amino acid residue are indicated on the right.

Thromboxane A2 Synthase Active Site

Fig. 1. Alignment of the partial amino acid sequences of TXAS with those of cytochromes P450BM-3, P450cam, and P450terp. The secondary structural elements of P450BM-3, P450cam, and P450terp are indicated by lines below the sequences. A, α helices; β, β-sheets; β strands. +, amino acid residues studied in this work. Numbers of amino acid residue are indicated on the right.

The use of the COS-1 expression system.

Ala-328 of P450BM-3 constitutes part of the hydrophobic pocket and is located in the vicinity of the heme moiety in the crystal structure (13). In the TXAS model (12), Ala-408 is superimposed on Ala-328 of P450BM-3. The loss of activity in the Ala-408 → Glu mutant suggested that Ala-408 is important for TXAS enzymatic activity (Table I and Fig. 3A). In the TXAS structural model (Fig. 2A), alteration of this alanine residue to glutamic acid can be envisioned as disrupting the substrate binding pocket required for interaction with hydrophobic region of PGH2. To test this hypothesis, we made additional, more conservative, mutations, changing the alanine residue to either glycine or leucine, which would be likely to maintain the hydrophobic environment of the substrate binding pocket. The Ala-408 → Gly and Ala-408 → Leu mutant constructs were expressed at levels similar to that of wild-type TXAS in the COS-1 cells (Fig. 3C). Additionally, the Ala-408 → Gly and Ala-408 → Leu mutants retained 30 and 44% of the wild-type enzymatic activities, respectively (Table I). The retention of TXAS activity in these conservative Ala-408 mutants supports our hypothesis that the loss of activity in the Ala-408 → Glu mutant was due to disruption of the hydrophobic environment rather than some general structural perturbation.

In the original structural model (Fig. 2A), Arg-413 did not appear to have any direct interactions with either the heme or bound PGH2. The complete loss of activity in the Arg-413 → Gly mutant (Table I) prompted us to test alternative binding orientations for PGH2. For this, the substrate was reoriented without changing the TXAS protein backbone structure, and a new round of energy minimization was performed. One resulting model, shown in Fig. 2B, suggests that Arg-413 could participate in substrate binding by forming a charge interaction with the carboxylate group of PGH2. This provides a plausible explanation for the loss of activity observed in the Arg-413 → Gly mutant. To test this model further, we altered the positively charged arginine residue at this position to either glutamine or lysine. As expected, mutation of Arg-413 to a neutral glutamine completely abolished enzymatic activity even though the recombinant protein was expressed at comparable levels (Fig. 3C and Table I). Interestingly, the positively charged Arg-413 → Lys mutant also lacked enzymatic activity, suggesting that the precise positioning of the positive charge is important.

Surprisingly, alteration of Arg-410 or Glu-433 to hydrophobic residues, changes that were anticipated from the TXAS model to disrupt substrate binding, produced only partial reduction in enzyme activity (Table I). To characterize further the effects of these mutations on TXAS catalysis, kinetic analyses of these mutant proteins were carried out in the presence or absence of U46619, a nonmetabolized PGH2 analog with moderate affinity for TXAS (29). The apparent Km and Vmax values of the wild-type and mutant proteins are summarized in Table II. The kinetic analysis indicated that the Arg-410 → Gly mutation did not greatly affect the apparent Km but increased the apparent Vmax value by 2-fold, confirming that U46619, as previously reported (29), is a competitive inhibitor. U46619 did not appreciably change the apparent Km value for the Arg-410 → Gly mutant. Glu-433 → Ala had an apparent Km that was approximately twice that of the wild type. The presence of U46619 decreased the apparent Km value for Glu-433 → Ala. These results suggest that the Arg-410 → Gly and Glu-433 → Ala mutants have altered affinity for PGH2 and/or its analog, consistent with the proximity of both residues to the TXAS active site in the structural model.
Fig. 2. Close-up stereo view of the substrate pocket and heme environment of the TXAS model. Note that PGH$_2$ is reoriented from the original (A) to the revised (B) model. Details are described in the text.
model (Fig. 2) to be located away from the substrate binding pocket, Thr-350, was also mutated. As expected, alteration of Thr-350 to Ala did not exert any influence on the TXAS protein level (Fig. 3A) or on the enzyme activity (Table I).

To address the possibility of altered product specificity, reversed-phase high performance liquid chromatographic analysis was carried out for products formed from [1-14C]PGH$_2$ by recombinant TXAS and several of the mutants, particularly those mutants that failed to synthesize detectable TXB$_2$. The wild-type recombinant enzyme produced both TXB$_2$ and 12-, hydroxy-5,8,10-heptadecatrienoic acid, as previously reported (23). No appreciable amounts of alternative products were found with the Ala-408 $\rightarrow$ Glu and Arg-413 $\rightarrow$ Gly mutants (data not shown), making it unlikely that the low apparent activity in these mutants was due to a shift in product profile.

Another important feature of the crystal structures of P450BM-3, P450cam, and P450terp is that helix I forms a hydrophobic backbone passing through the center of the molecule and creates an unusual groove adjacent to the heme region (13, 28). The helix I residue corresponding to Thr-252 in P450cam is noteworthy that TXAS, unlike other P450s, catalyzes scission of molecular oxygen for a monooxygenation reaction. Epidioxy cleavage requires less energy for a peroxide than for a dioxygen molecule (33) and is thus less likely to be the rate-limiting step in TXAS biosynthesis. This may explain why threonine or serine is not

![Image](https://example.com/image.png)

**Fig. 3. Western blot analysis of TXAS in microsomal preparations from COS-1 cells transfected with wild-type and mutant TXAS cDNAs.** Microsomal proteins (30 μg) from human platelets or those mutants that failed to synthesize detectable TXB$_2$. The wild-type recombinant enzyme produced both TXB$_2$ and 12-, hydroxy-5,8,10-heptadecatrienoic acid, as previously reported (23). No appreciable amounts of alternative products were found with the Ala-408 $\rightarrow$ Glu and Arg-413 $\rightarrow$ Gly mutants (data not shown), making it unlikely that the low apparent activity in these mutants was due to a shift in product profile.

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**TABLE I**

| Construct | TXAS activity$^a$ | Relative activity$^a$ |
|-----------|------------------|----------------------|
| Wild type (n = 10)$^c$ | 880 ± 280 | 100 |
| Vector (n = 7) | 48 ± 30 | 5 |
| Ala-408 $\rightarrow$ Glu (n = 1) | 60 | 7 |
| Ala-408 $\rightarrow$ Gly (n = 2) | 257 | 29 |
| Ala-408 $\rightarrow$ Leu (n = 2) | 385 | 44 |
| Arg-413 $\rightarrow$ Gly (n = 2) | 36 | 4 |
| Arg-413 $\rightarrow$ Ile (n = 2) | 45 | 5 |
| Arg-413 $\rightarrow$ Glu (n = 2) | 42 | 5 |
| Arg-410 $\rightarrow$ Gly (n = 5) | 430 ± 120 | 49 |
| Glu-433 $\rightarrow$ Ala (n = 2) | 320 | 36 |
| Asn-110 $\rightarrow$ Ile (n = 2) | 52 | 6 |
| Phe-127 $\rightarrow$ Val (n = 1) | 62 | 7 |
| Arg-478 $\rightarrow$ Ala (n = 5) | 35 ± 10 | 4 |
| Cys-480 $\rightarrow$ Ser (n = 3) | 36 ± 20 | 4 |
| Ile-346 $\rightarrow$ Thr (n = 1) | 1230 | 139 |
| Thr-350 $\rightarrow$ Ala (n = 1) | 740 | 84 |

$^a$ Average, or average ± S.D. presented for replicate transfections.

$^b$ To correct for batch-to-batch variations in transfection efficiency, the activity of each mutant is normalized to the wild-type activity in the same transfection experiment.

$^c$ The numbers of separate transfection experiments are given in parentheses.

![Image](https://example.com/image.png)

**TABLE II**

Kinetic analyses of TXAS catalysis by microsomal preparations of recombinant wild-type and mutant TXAS

To minimize variability, the kinetic parameters presented in this table were from a single batch of COS-1 cells transfected at the same time. Western blot analysis verified that similar levels of expression were obtained for the wild-type and mutant proteins.

| Construct | $K_m$ protein/min | $V_{max}$ pg TXB$_2$/μg protein/min | $K_m$ protein/min | $V_{max}$ pg TXB$_2$/μg protein/min |
|-----------|------------------|-------------------------------------|------------------|-------------------------------------|
| Wild-type | 4.6 ± 1.0 850 ± 80 | 10.4 ± 0.8 720 ± 30 | | | | |
| Arg-410 $\rightarrow$ Gly | 6.0 ± 2.0 230 ± 30 | 7.0 ± 1.0 190 ± 20 | | | | |
| Glu-433 $\rightarrow$ Ala | 11 ± 3 200 ± 30 | 5.0 ± 1.0 140 ± 10 | | | | |
prerequisite for O–O bond scission in TXAS as it is for the P450 monooxygenase enzymes.

Heme Environment—Two amino acid residues in the heme binding region are conserved among all P450s: a cysteine residue, which serves as the proximal axial ligand for the heme iron through a thiolate bond, and an arginine or a histidine residue, which forms a hydrogen bond with the D-ring propionate group of the heme moiety. In TXAS, these residues correspond to Cys-480 and Arg-478. Our molecular modeling, as shown in Fig. 2B, also predicted that Arg-413 could act as a bridge, stabilizing substrate binding. As in the original model, Cys-480, Arg-478, Phe-127, and Asn-110 are in the immediate vicinity of the heme center. It is interesting to note that greatly decreased expression was also found for three other mutations of residues predicted to participate in binding the TXAS heme: Asn-110 to Ile, Phe-127 to Val, and Asn-110 to Ilebysite-directed mutagenesis (Fig. 2B). The low amounts of recombinant TXAS and human platelet microsomes. The low amounts of residues predicted to participate in binding the TXAS heme: Asn-110 to Ile, Phe-127 to Val, and Arg-478 to Ala (Fig. 3B). Misfolded proteins are known to be susceptible to accelerated degradation in the endoplasmic reticulum compartment of mammalian cells (35). It may be that any disruption of proper heme binding in TXAS alters the overall protein folding and targets the misfolded protein for degradation by endogenous proteases.

Conclusion—Our revised three-dimensional structure of the TXAS model (Fig. 2B) predicts that Arg-413 can act as a bridge, interacting with Glu-433 and the carboxylate group of PGH2. The revised orientation of PGH2 also puts Arg-410 closer to the center of the PGH2 molecule, where this arginine residue might participate in the catalytic reaction of TXA2 formation. Ala-408 is also located near PGH2 in the new orientation and could plausibly contribute to the hydrophobic environment to stabilize substrate binding. As in the original model, Cys-480, Arg-478, Phe-127, and Asn-110 are in the immediate vicinity of the heme moiety. Cys-480, the thiolate ligand of the TXAS heme iron, is located on the heme face away from the substrate binding pocket. Arg-478 is predicted to play an important role in maintaining the heme in its proper environment through a charge interaction with the propionate. In the revised model, Phe-127 is not only adjacent to the heme propionate ring but also close to the bound substrate. Furthermore, the C-9 endoperoxide oxygen atom of PGH2 is oriented toward the heme iron, in accordance with the spectroscopic data indicating an interaction between this oxygen and the heme iron (36).

The results of the present mutagenesis study support the general features of the active site in the TXAS structural model and have led to refinement of the substrate binding site. The revised TXAS model should be useful for further characterization of the active site structure and function of this unusual cytochrome P450.

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