Expression, Purification, and Spectroscopic Characterization of Human Thromboxane Synthase*

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Thromboxane A₂ (TXA₂) is a potent inducer of vasocostriction and platelet aggregation. Large scale expression of TXA₂ synthase (TXAS) is very useful for studies of the reaction mechanism, structural/functional relationships, and drug interactions. We report here a heterologous system for overexpression of human TXAS. The TXAS cDNA was modified by replacing the sequence encoding the first 28 amino acid residues with a CYP17 amino-terminal sequence and by adding a polyhistidine tag sequence prior to the stop codon; the cDNA was inserted into the pCW vector and co-expressed with chaperonins groES and groEL in Escherichia coli. The resulting recombinant protein was purified to electrophoretic homogeneity by affinity, ion exchange, and hydrophobic chromatography. UV-visible absorbance (UV-Vis), magnetic circular dichroism (MCD), and electron paramagnetic resonance (EPR) spectra indicate that TXAS has a typical low spin cytochrome P450 heme with an oxygen-based distal ligand. The UV-Vis and EPR spectra of recombinant TXAS were essentially identical to those of TXAS isolated from human platelets, except that a more homogenous EPR spectrum was observed for the recombinant TXAS. The recombinant protein had a hemeprotein molar ratio of 0.7:1 and a specific activity of 12 μmol of TXA₂/min/mg of protein at 23 °C. Furthermore, it catalyzed formation of TXA₂, 12-hydroxy-5,8,10-heptadecatrienoic acid, and malondialdehyde in a molar ratio of 0.94:1.0:0.93. Spectral binding titrations showed that bulky heme ligands such as clotrimazole bound strongly to TXAS (Kₐ = 0.5 μM), indicating ample space at the distal face of the heme iron. Analysis of MCD and EPR spectra showed that TXAS was a typical low spin hemoprotein with a proximal thiolate ligand and had a very hydrophobic distal ligand binding domain.

Thromboxane A₂ synthase (TXAS) converts prostaglandin H₂ (PGH₂) to TXA₂, which is a vasoconstrictor and a potent stimulus of platelet secretion and aggregation (1). TXA₂ is rather labile, being hydrolyzed with half-life of about 30 s in aqueous solution to the biologically inactive thromboxane B₂ (2). This lability indicates that TXA₂ is primarily an autocrine or paracrine mediator, acting in the vicinity of its biosynthesis.

TXAS was characterized spectrophotometrically as a cytochrome P450 enzyme and was found to be associated with the endoplasmic reticulum (3, 4). Unlike other microsomal P450s that require a P450 reductase as electron donor to catalyze a mono-oxygenation reaction, TXAS catalyzes an isomerization reaction without need for a reductase or other electron donor or for molecular oxygen. Interestingly, TXAS also catalyzes formation of the scission products, 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and malondialdehyde (MDA) at a ratio of 1:1:1 with TXA₂ (3).

Although TXAS was purified from platelets more than a decade ago, limited information has been obtained about the reaction mechanism or about structural/functional relationships. This is mainly due to the low abundance of the enzyme (0.1% of platelet microsomal protein) and the difficulty in purifying sufficient enzyme for kinetic and spectroscopic studies. Conventional chromatography of platelet TXAS was found to have poor yields and reproducibility (3). Immunoaffinity purification of TXAS was accompanied by loss of >90% of the activity during elution (5, 6). TXAS purified on a high pressure liquid chromatographic, anion exchange column lost its P450 characteristics and became an inactive P420 form (7). The outlook has been improved by isolation of the TXAS cDNA (8–12). Several groups have described systems for heterologous expression of TXAS. In prokaryotic expression systems, fusion of TXAS with glutathione S-transferase produced a protein of the correct size but that was enzymatically inactive (4); insertion of TXAS cDNA into a common P450 expression vector, pCW, resulted in a very low yield of recombinant protein (13). In eukaryotic expression systems, plasmid-directed transient expression in COS-1 cells and baculovirus-driven expression in SF9 cells generated enzymatically active TXAS but in amounts insufficient for spectroscopic studies (4, 10, 14). Here, we report development of a high level bacterial expression system for human TXAS and a rapid and reproducible purification procedure that affords milligram levels of active enzyme suitable for detailed biophysical and biochemical characterization.

EXPERIMENTAL PROCEDURES

Materials—Nickel-nitritolatriacetate agaroce (Ni-NTA) was obtained from Qiagen. DEAE-Sephacel and octyl-Sepharose were from Amersham Pharmacia Biotech. PGH₂ was purchased from Biomol, and the stable PGH₂ analog, U44069 (15-hydroxy-9,11-[epoxymethano]prosta-5,13-dienoic acid), was from Cayman. Imidazole, 1-phenylimidazole, 1-butylimidazole, 2-phenylimidazole, 4-phenylimidazole, pyridine, 5,13-dienoic acid), was from Cayman. Imidazole, 1-phenylimidazole, 1-butylimidazole, 2-phenylimidazole, 4-phenylimidazole, pyridine, 4-ethylpyridine, 3,5-lutidine, pyrimidine, 2-aminopyrimidine, and 4-ethylpyridine were from Aldrich. Clotrimazole (1-[2,4-dichlorophenyl]-2,4-dihydropyridyl) and miconazole (1-[2,4-dichlorophenyl]-2,4-dihydropyridyl) were obtained from Sigma. Emulgen 913 was a gift from Kao Chemicals, Tokyo, Japan. The pCW and pT-groE expression vectors were kindly provided by Dr. Amy Roth.

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5 The abbreviations used are: TXAS, thromboxane A₂ synthase; TXA₂, thromboxane A₂; PGH₂, prostaglandin H₂; HHT, 12-1-hydroxy-5,8,10-heptadecatrienoic acid; MDA, malondialdehyde; Ni-NTA, nickel-nitritolatriacetate agaroce; MCD, magnetic circular dichroism.
The sequences of sense primers (in 5' to 3' direction) for the modified amino termini are as follows: AACATATGGCTCTGTTATTAGCAGTTTTT (Ndel site is underlined), immediately followed by TXAS cDNA sequences of the desired truncations. The sequences of antisense primers (in 5' to 3' direction) are as follows: GAAGATCTCAGTGATGGTGATGGCGGGATACGATCTTGATAT (for constructs with a four-histidine tag) or GAAGATCTAACATCCACACTTAGGGT (for constructs with native carboxyl terminus; the sequence corresponds to 49–66 bases downstream from the TGA stop codon) (BglII sites are underlined). The resultant amino acid sequences are shown below. The cDNAs were constructed into the pcW expression vector and transformed into JM109 cells. After induction with isopropyl-1-thio-β-d-galactopyranoside, cells were sonicated and the homogenate was centrifuged at 10,000 × g for 10 min. Proteins in the supernatant were separated by SDS-polyacrylamide gel electrophoresis, and the TXAS expression levels were estimated by Coomassie staining and immunoblot analyses.

| Construct | Amino acid sequence | Relative expression level |
|-----------|---------------------|--------------------------|
| Wild type | MMEALGFKLKEVN...IVSR | +                        |
| (Δ-2)mod  | MALLAVFALLAL...IVSR  | +++                     |
| (Δ-12)mod| MALLAVFALLALL...IVSRHHHH | +++                  |
| (Δ-28)mod| MALLAVFALLAL...IVSRHHHH | +++                   |
| (Δ-59)mod| MALLAVFALLAL...IVSRHHHH | +++                   |
| (Δ-65)mod| MALLAVFIMESQV...IVSRHHHH | +++               |

(University of Oregon) and Dr. Shunsuke Ishii (Institute of Physical and Chemical Research, Ibaraki, Japan), respectively.

Construction of TXAS Expression Plasmids—The polymerase chain reaction was used to amplify the human TXAS cDNA, isolated from a human lung cDNA library (8). The sense primers were designed to introduce the initiator codon ATG within an Ndel site and to make desired truncations of the TXAS amino-terminal region, e.g., by replacing amino acid residues 1–28 with an 8-residue segment (MALLLAVF) favoring expression in Escherichia coli (15). The antisense primers were designed to add a BglII site near the carboxyl terminus, with or without a 4-histidine terminal segment to facilitate protein purification. Individual polymerase chain reaction products were digested with NdeI and BglII and ligated into the corresponding restriction enzyme sites of the pcW plasmid, which uses the tac promoter to drive expression (16). The resultant amino- and carboxyl-terminal amino acid sequences of recombinant TXAS are listed in Table I. Plasmids were transformed into a protease-deficient E. coli strain BL21 (DE3) containing the chloramphenicol-resistant plasmid pGroE (17), which expresses chaperonins GroES and GroEL from the T7 promoter. Colonies were isolated by LB-agar plates containing both ampicillin and chloramphenicol. A typical overnight culture (20 ml) was inoculated into 1 liter of LB medium with 100 μg/ml ampicillin and 25 μg/ml chloramphenicol. Bacteria were grown at 37 °C in a shaker at 200 rpm until the Klett reading reached 100–140. Isopropyl-β-D-thiogalactopyranoside (1 mM) and d-aminolevulinic acid (0.5 mM) were then added, and the culture was continued at 160 rpm for 20 h at 30 °C before harvesting. The pelleted cells were kept at −70 °C until protein purification.

Cultured cells (17 g from 5 liters of culture medium) were resuspended in 90 ml of Buffer A (0.1 mM sodium phosphate, pH 7.5, 10% glycerol, and 0.1 mM NaCl) containing 50 μg/ml DNase, 2 mM MgCl2, 100 units/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride and lysed by two passes through a French pressure cell at 3000 psi. The suspension was then centrifuged at 10,000 × g for 30 min, and the supernatant containing solubilized TXAS was diluted to 200 ml with Buffer A and applied to a Ni-NTA column (1.5 × 9 cm) pre-equilibrated with Buffer A. The column was washed with 5 column volumes of Buffer A, then with 10 column volumes of Buffer B (0.02 M sodium phosphate, pH 7.5, 0.02% of Emulgen 913, and 10% glycerol) containing 5 mM histidine, and then with 5 column volumes of Buffer B containing 10 mM histidine to remove nonspecifically bound proteins. TXAS was eluted with Buffer B containing 40 mM histidine. The brown-colored fractions were pooled and loaded on a DEAE-Sephacel column (1.5 × 3 cm) pre-equilibrated with Buffer B. The flow-through fractions, which contained the majority of the TXAS activity, were collected and applied to an octyl-Sepharose column (1.5 × 3 cm) pre-equilibrated with Buffer C (0.02 M sodium phosphate, pH 7.5, and 10% glycerol) containing 0.5 mM NaCl. The column was washed with 10 volumes of the same buffer and then with 10 volumes of Buffer C. TXAS was eluted with Buffer C containing 0.2% Emulgen 913 and stored at −70 °C until used.

The level of recombinant TXAS expression was assessed by Coomassie Blue staining after separation of proteins by polyacrylamide gel electrophoresis under denaturing conditions; the identity of TXAS was confirmed by Western blot analysis using antiserum raised against a glutathione transferase-TXAS fusion protein (4).

Heme and Protein Assays—Heme content was determined by the formation of pyridine hemochromogen (18). Briefly, the purified TXAS (150 μg) was incubated in 0.15 M NaOH and 1.8 mM pyridine and then reduced by addition of a few drops of dithionite. Absorbance differences at 556 nm and 540 nm were recorded for the reduced and oxidized heme pyridine complexes. A difference extinction coefficient of 24 mM⁻¹ cm⁻³ (reduced minus oxidized forms) was used for calculation of heme concentration. Protein concentrations were determined by bichonichic acid assay using bovine serum albumin as standard (19). TXAS was also quantified for total number of tryptophan residues by magnetic circular dichroism (MCD) using L-tryptophan as standard (A280 = 5500 M⁻³ cm⁻¹) (20).

Enzyme Assay—TXAS activity was routinely measured by a coupled assay as follows. Purified sheep prostaglandin H synthase (about 1000 units; Ref. 21) was mixed with TXAS in 190 μl of 30 mM Tris, pH 7.5, containing 1 μl hemin at 23 °C. The reaction was initiated by adding 10 μl of 12.5 mM arachidonic acid in (ethanol and continued for 3 min before stopping with 5 μl of 2 N HCl. The reaction mixture was neutralized with 20 μl of 1 M Tris, pH 8.0, prior to the radioimmunoassay for thromboxane B₂ (22), the stable hydration product of TXA₂. To obtain Kₐ and Vₐ values, PGH₂ in a small volume of isopropyl alcohol was added directly to TXAS in 30 mM Tris, pH 7.5.

UV-Vis, MCD, and EPR Spectroscopy—UV-Vis absorbance spectra were recorded with an HP8452 diode array spectrophotometer or a Shimadzu UV-2401PC spectrophotometer. Reduced CO difference spectra were obtained by treating the protein with dithionite and then purging the sample cuvette with carbon monoxide. Heme ligand perturbation spectra were obtained by titration of the protein with a concentrated stock solution of ligand. Dissociation constants (Kₐ) were determined by fitting the peak-trough amplitudes from the perturbation difference spectra and the corresponding ligand concentrations to a one-site hyperbolic binding model (23).

MCD spectra were obtained at room temperature using a Jasco J-500C spectrometer with a 1.3 Tesla electromagnet calibrated with camphor sulfonic acid and potassium ferricyanide (24). EPR spectra were recorded at liquid helium temperature on a Varian E-4 spectrometer with an Air Products liquid helium transfer line (25). EPR parameters, including g values, V, and D, the rhombic and axial ligand field terms for low spin heme complexes, and the ratio of rhombic (E) and axial (D) ligand field components for high spin heme complexes, were determined as described previously (25).

RESULTS

Optimization of Expression Plasmid—Expression of human TXAS in E. coli was assessed for various TXAS cDNA constructs. Initially, full-length, wild-type TXAS cDNA was ligated into the pcW vector, a system that has been widely used in the heterologous bacterial expression of other P450s (26). Coomassie staining and immunoblot analysis indicated that the full-length construct yielded low levels of expression in E. coli (Table I), similar to results with other unmodified P450 cDNAs (27). In light of earlier results with P450c17 expression in E. coli (28), we modified the TXAS cDNA by progressive truncation of the amino-terminal sequence and insertion of the
TABLE II

Purification of recombinant TXAS from E. coli

| Step                  | Protein | Specific activity | Yield |
|-----------------------|---------|-------------------|-------|
| Homogenate            | 2670    | 29.7              | 100   |
| Detergent extract     | 1780    | 38.1              | 87    |
| Ni-NTA column         | 18.4    | 1090              | 26    |
| DEAE column           | 4.64    | 2800              | 17    |
| Octyl column          | 3.72    | 2950              | 14    |

The heme content of the purified recombinant TXAS was calculated to be 0.94:1.0:0.93, consistent with published results using purified platelet TXAS (3). The small fraction of 420 nm species in the reduced heme-CO spectrum in Fig. 3 is probably not an inactive P420 form of TXAS because complete conversion to the heme-carbon monoxide complex of dithionite-reduced TXAS had a major peak at 450 nm, and minor peaks at 420 nm and 546 nm, similar to the reduced CO spectrum reported for purified platelet TXAS (3). The molar ratio of TXA2, HHT, and MDA formed by the recombinant TXAS was calculated to be 0.94:1.0:0.93, consistent with published results using purified platelet TXAS (3).

Characterization of Recombinant TXAS UV-Vis and MCD Spectra—The heme content of the purified recombinant TXAS determined by pyridine-hemochromogen analysis was 0.72 ± 0.06 mol of heme/mol of protein, indicating that some 70% of the purified recombinant protein was present as the holoenzyme. This is not due to the inaccuracy of protein quantitation since results from bicinchoninic acid assay and noninvasive MCD quantitation for tryptophan residues were consistent. The purified TXAS exhibited an electronic spectrum typical for low spin cytochrome P450, with a Soret maximum at 418 nm and α and β bands at 567 and 555 nm, respectively (Fig. 3). The heme-carbon monoxide complex of dithionite-reduced TXAS had a major peak at 450 nm, and minor peaks at 420 nm and 546 nm, similar to the reduced CO spectrum reported for purified platelet TXAS (3). The small fraction of 420 nm species in the reduced heme-CO spectrum in Fig. 3 is probably not an inactive P420 form of TXAS because complete conversion to the 450 nm species was observed in the presence of methyl viologen, a mediator dye (data not shown). The difference spectrum between the reduced heme-CO complex and resting TXAS highlights the 450 nm peak, characteristic of cytochrome P450 (Fig. 3).

To evaluate the effects of axial ligands on the TXAS heme behavior, UV-Vis and MCD spectroscopy were used to charac-
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Fig. 2. Conversion of PGH₂ to HHT and MDA by TXAS. Both sample and reference cuvettes contained purified TXAS (8 pmol) in 0.3 ml of 30 mM Tris, pH 7.5, at 23 °C. The reaction was initiated by addition of 4 μl of 1.4 mM PGH₂ to the sample cuvette. Absorption spectra were recorded at 1 (○), 2 (□), 3 (▴), and 5 min (▼) after the initiation of reaction.

Fig. 3. Electronic absorption spectra of recombinant TXAS. Purified TXAS (3 μg) was incubated in 0.3 ml of 20 mM sodium phosphate, pH 7.5, containing 10% glycerol and 0.2% Emulgen 913 (- - -). A few crystals of dithionite were added to the enzyme sample before it was purged with CO gas for 1 min (---). The difference between resting and reduced-CO spectra is also shown (-----).

terize resting TXAS and TXAS complexed with U44069 (O-based ligand) or imidazole (N-based ligand). As shown in Fig. 4A, resting TXAS had optical absorbance peaks at 360 (46.2 mM⁻¹ cm⁻¹), 418 (100 mM⁻¹ cm⁻¹), 535 (13.6 mM⁻¹ cm⁻¹), 567 (12.3 mM⁻¹ cm⁻¹), and 655 (4.9 mM⁻¹ cm⁻¹) nm. The U44069 complex exhibited a blue-shifted Soret peak at 414 nm (101 mM⁻¹ cm⁻¹) and bands in the visible region at 531.5 (12.7 mM⁻¹ cm⁻¹), 563 (12.5 mM⁻¹ cm⁻¹), and 652 (4.7 mM⁻¹ cm⁻¹) nm. In contrast, imidazole ligation of the TXAS yielded a bathochromic shift in the electronic spectrum, with peaks at 424 (97.4 mM⁻¹ cm⁻¹) and 540 (4.4 mM⁻¹ cm⁻¹) nm. These spectra are typical for low spin P450 hemoproteins in both peak positions and amplitude (30). The MCD spectra for TXAS shown in Fig. 4B display standard features typical for a low spin P450 heme with the symmetric Soret crossovers closely matching the electronic absorbance peaks. The two marker MCD bands at 566.1 and 520.7 nm in the visible region for resting TXAS and its complex with imidazole (560.7 and 526.5 nm) or U44069 (549.9 and 515.1 nm) are very similar to those seen with low-spin P450cam, chloroperoxidase (31), and nitric oxide synthase (32, 33), each of which has a proximal thiolate ligand. The lack of significant features in the 600–700 nm region of the MCD spectrum (Fig. 4B) indicated that the TXAS complexes were primarily low spin in nature.

Characterization of Recombinant TXAS EPR Spectra—X-band EPR spectra of TXAS derivatives were measured at liquid helium temperature. Except for some nonspecific signals at g = 4.3 (adventitious iron) and at g = 2 (organic radicals), we only observed signals for low spin heme. Detailed EPR spectra in the low spin heme region for TXAS and its complexes with imidazole and U44069 are shown in Fig. 5. The rhombic heme species have g tensor values of 2.419, 2.252, and 1.918 for resting TXAS (spectrum B) and of 2.464, 2.255 and 1.894 for the imidazole derivative (spectrum A), indicating the presence of a single low spin heme species in both resting TXAS and its complex with imidazole. The g values observed for resting enzyme are very similar to those reported for platelet TXAS (3), although the platelet enzyme showed two EPR species with slightly different rhombicity. At saturating levels of U44069, the recombinant TXAS heme did show two EPR species (Fig. 5, spectrum C); one exhibited the spectral features of resting enzyme, whereas the other appeared to be a complex with the ligand. Subtracting 36% of the amplitude of spectrum B from spectrum C reveals a new EPR species having g values of 2.484, 2.252, and 1.900 (Fig. 5, spectrum D). The closeness in g values between O-based ligand (U44069) in spectrum D and N-based ligand (imidazole) in spectrum A indicates that the TXAS EPR is not very sensitive to the nature of the TXAS distal heme ligand. Moreover, the lack of complete conversion to the rhombic heme species even with excess U44069 implies that a portion of the enzyme (~36%) is inaccessible to this ligand. These results parallel our finding that a small fraction of the TXAS is impaired for reduction and CO complex formation (Fig. 3). The resting TXAS heme EPR exhibited a heterogenous saturation behavior with half-saturation power at 75 microwatts, similar to values reported for other low spin cytochrome P450s (Fig. 6A; Ref. 34).

The EPR characteristics of resting, imidazole-, and U44069-bound TXAS fit nicely into the "P" zone of the "Truth diagram" correlating the low spin heme rhombicity and tetragonal field strength, as originally devised by Blumberg and Peisach (Fig. 6B; Ref. 35), indicating that TXAS has a typical P450 electronic
structure with a proximal thiolate ligand. In addition, TXAS and its complexes with imidazole and U44069 fall in a region within P zone overlapping with P450cam but not chloroperoxidase or nitric oxide synthase.

Comparison of TXAS Interactions with Various Heme Ligands—The difference spectrum obtained upon addition of U44069 to TXAS exhibited a maximum at 409 nm and a trough at 426 nm (data not shown). A similar result was obtained for platelet TXAS (36) and interpreted to mean that U44069 displaced the original heme ligand (probably water), with the C-9 oxygen atom of U44069 interacting with the heme iron to form a six-coordinate complex. The dissociation constant of the complex of recombinant TXAS with U44069 was estimated by a spectral perturbation method (see “Experimental Procedures”) to be 28 ± 4 μM at 23 °C. To characterize further the structure of distal heme site in recombinant TXAS, we extended our spectral perturbation studies to other heme ligands that have a nitrogen atom as the distal heme ligand but that differ in the size and position of substituent groups. The ligands were divided into three groups as follows: imidazole-based, pyridine-based, and pyrimidine-based ligands (Table III). All the ligands produced a type II spectral change, with Soret peaks around 420–430 nm, an indication that the exogenous ligand replaced the native distal ligand and formed a six-coordinate complex (37). The imidazole-based ligands showed a wide range of affinities for TXAS, with $K_d$ values ranging from 0.5 μM for clotrimazole to more than 25 μM for 2-phenylimidazole. Interestingly, bulky and hydrophobic derivatives, such as clotrimazole and miconazole, bound strongly to TXAS. Preliminary molecular modeling using Alchemy III software (Tripos Associates, Inc., St. Louis, MO) was conducted to assess the geometry of the rigid imidazole analog, clotrimazole. The tetrahedral carbon at the imidazole N-1 position is bonded with three phenyl rings making clotrimazole a very rigid molecule, with...
vertical and horizontal dimensions of ~8.5 and 9.5 Å. This provides approximate minimal dimensions of the TXAS distal heme pocket. The results in Table III suggest that the TXAS distal heme pocket can accommodate relatively bulky substitu-
ents at the 1-position of imidazole. On the other hand, the lower affinity of 4-imidazole suggests that the distal heme pocket has less space to accommodate substituents at the 4-position. The very much lower affinity for 2-phenylimidazole is likely due to steric hindrance between the bulky 2-substituent and heme porphyrin ring. The three pyridine-based ligands tested had moderate affinities for the TXAS heme, with $K_d$ values of 40–180 μM (Table III). All three pyrimidine-based ligands tested had quite low affinity for the TXAS heme with $K_d$ values of 1.9–12.7 μM (Table III), consistent with pharmacological studies with platelet microsomes which found that pyrimidine was a poor TXAS inhibitor (38).

**DISCUSSION**

A common strategy for prokaryotic expression of mammalian P450s involves replacement of the natural P450 amino termi-
nus with the MALLLAVF sequence derived from bovine steroid 17α-hydroxylase, CYP17 (26). This approach has been used successfully to express in E. coli of several other P450s, including CYP1A2 (39), CYP3A4 (40), CYP55 (41), and several members of the CYP2C subfamily (42, 43). A previous attempt to express TXAS in E. coli without the modified CYP17 amin-
terminal sequence (13) resulted in a much lower expression level than the present system (Table I), indicating that an amino-terminal sequence such as MALLLAVF promotes high level expression of recombinant TXAS. Furthermore, variation of the extent of truncation at the TXAS amino-terminal had profound effects on TXAS expression (Table I), indicating that amino-terminal sequences beyond the modified CYP17 segment are also important in achieving a high level of TXAS expression. Although the expression level with the modified amino-terminal sequence was high, the majority of recombi-
nant TXAS synthesized in E. coli was still enzymatically inac-
tive and remained in inclusion bodies. Co-expression of TXAS with bacterial chaperonins, a strategy used with neuronal ni-
tric oxide synthase (44), did improve the yield of active TXAS, indicating that the post-translational maturation process may be limiting in the bacterial expression system.

The purified recombinant TXAS shows some interesting het-
erogeneity in its interactions with reductant and substrate analog. Upon reduction in the presence of CO, about 30% of the material retained an absorbance peak at 420 nm instead of shifting to 450 nm (Fig. 3); a small fraction of 420 nm species was also observed with purified platelet TXAS (3). The 420 nm species in TXAS was readily converted to the 450 nm form by addition of the electron transfer mediator, methyl viologen, implying that there is a barrier to electron transfer from di-
thionite in about one-third of the recombinant TXAS molecules. A similar fraction of recombinant TXAS appeared unable to bind U44069 (Fig. 5). Because 70% of the purified TXAS preparation contains heme and thus contributes to the Soret absor-
bance, we estimate that just about half (0.7 ± 0.7 ± 0.49) of the purified recombinant TXAS is involved in catalysis. This leads to an estimated intrinsic specific activity of 24 μmol of TXA$_2$/min/mg of protein. This is almost exactly the specific activity reported for purified platelet enzyme (24.1 μmol of TXA$_2$/min/mg of protein; Ref. 3). Compared with most microso-
mal P450 monoxygenases, which have specific activities of only nmol of product/min/mg of protein (45), TXAS can be seen as a relatively efficient catalyst. It should also be noted that recombinant TXAS, like the purified platelet enzyme, catalyzes formation of TXA$_2$ along with HHT and MDA in approximately a 1:1:1 ratio. The physiological roles of HHT and MDA are not
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The similarity between the heme EPR properties of TXAS and P450$_{cam}$, and their differences with chloroperoxidase and nitric oxide synthase derivatives in the P zone of Fig. 6B indicate that the polarity of the axial heme ligand pockets in TXAS and P450$_{cam}$ are less polar than in chloroperoxidase or nitric oxide synthase. This fits with the relative substrate polarities in these enzymes, as the substrates for chloroperoxidase and nitric oxide synthase are polar in nature, whereas the substrates for P450$_{cam}$ and TXAS are relatively hydrophobic.

Pharmacological TXAS inhibitors have been actively sought because of the anticipated beneficial effects of reducing platelet activation at the site of acute vascular lesions. Existing TXAS inhibitors can be divided into three structural groups as follows: substrate analogs, imidazole-based inhibitors, and pyridine-based inhibitors (48–50). The dissociation constants of some of these inhibitors measured with purified recombinant TXAS (Table III) correlate well with drug potency assayed using platelet microsomes (38, 48–50), confirming that the purified, detergent-solubilized TXAS is functionally similar to the membrane-bound form. This indicates that recombinant TXAS will be useful for detailed characterization of enzyme interactions with potential inhibitors.

In summary, catalytically active human TXAS has been expressed in E. coli, and a rapid and inexpensive purification protocol was developed to provide the quantities of enzyme required for physiochemical characterization, reaction mechanism studies, and inhibitor design. Initial spectroscopic characterization of recombinant TXAS indicates the distal heme pocket is hydrophobic and relatively large compared with “classical” P450 enzymes.

**TABLE III**

Spectral perturbation studies of the TXAS heme environment

| Ligand                  | Difference spectrum features | $K_d$ |
|-------------------------|------------------------------|-------|
|                         | peak, nm                     | trough, nm | $\mu M$ |
| **Imidazole derivatives** |                              |       |       |
| Imidazole               | 434                          | 412    | 33 ± 2 |
| 1-Phenylimidazole       | 430                          | 400    | 37 ± 4 |
| 1-Butylimidazole        | 432                          | 396    | 2.0 ± 0.2 |
| 2-Phenylimidazole       | 432                          | 410    | 180 ± 10 |
| 4-Phenylimidazole       | 432                          | 410    | 0.47 ± 0.07 |
| Clotrimazole            | 431                          | 410    | 1.5 ± 0.2 |
| Miconazole              | 430                          | 397    |       |
| **Pyridine derivatives** |                              |       |       |
| Pyridine                | 428                          | 392    | 57 ± 9 |
| 4-Ethylpyridine         | 428                          | 392    | 41 ± 2 |
| 3,5-Lutidine            | 430                          | 396    | 176 ± 8 |
| **Pyrimidine derivatives** |                            |       |       |
| Pyrimidine              | 422                          | 388    | 3,700 ± 1,000 |
| 4-                        | 424                          | 391    | 1,900 ± 150 |
| Methylpyrimidine        |                              |       |       |
| 2-Aminopyrimidine       | 425                          | 396    | 13,000 ± 3,000 |

**Fig. 6.** EPR signal characteristics of resting TXAS and its complexes with imidazole and U44069. A, power saturation. The signal amplitudes (solid circles) are shown as a function of the microwave power. The curve shows the result of fitting to the inhomogeneous saturation function (Equation 1):

$$\log (S/P_{1/2}) = -b/2 \log (P_{1/2} + P) + b/2 \log (P_{1/2}) + \log (K)$$  (Eq. 1)

where $P_{1/2}$ is the power to achieve half-saturation of the signal, and $K$ is a proportionality factor; the value of $b$ is set to 1 for inhomogeneous broadening as found for most hemeproteins (25). $B$, truth diagram analysis. The rhombicity values ($V/\Delta$) are plotted as a function of the tetragonal field values ($\Delta/\lambda$) for TXAS and its derivatives with imidazole and U44069 (open triangles). The six zones indicate the assignment reported previously (25). Complexes in C zone are methionine and histidine ligands; in B zone are bis-histidine or bis-imidazole ligands; in $H$ zone are histidine/imidazole or histidine/azide ligands; in O zone are histidine/hydroxide or histidine/tyrosinate ligands; in P zone are proximal thiolate ligands and in CN$^-$ zone are cyanide derivatives with proximal ligands other than thiolate. Data for P450$_{cam}$ (filled squares), chloroperoxidase (filled circles), and endothelial nitric oxide synthase (open diamonds) are included for comparison.

The similarity between the heme EPR properties of TXAS and P450$_{cam}$, and their differences with chloroperoxidase and nitric oxide synthase derivatives in the P zone of Fig. 6B indicate that the polarity of the axial heme ligand pockets in TXAS and P450$_{cam}$ are less polar than in chloroperoxidase or nitric oxide synthase. This fits with the relative substrate polarities in these enzymes, as the substrates for chloroperoxidase and nitric oxide synthase are polar in nature, whereas the substrates for P450$_{cam}$ and TXAS are relatively hydrophobic.

Despite the fact that TXAS is a non-classical P450 and catalyzes an isomerization reaction rather than monooxygenation, a typical low spin P450 heme state was observed for TXAS in both absorption and EPR spectroscopies. MCD is more reliable than EPR in assigning the axial heme ligand for P450-type hemeproteins (47). Due to the dominant effect of the thiolate proximal ligand in comparison with $N$- or $O$-based distal ligand, the P450 $g$ values measured by EPR are rather insensitive to the nature of the axial heme ligand, leaving the values of EPR parameters for various P450s clustered in a very small region (47). In contrast, MCD spectra display distinctive sig-
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