Expression of two different forms of cDNA for thromboxane synthase in insect cells and site-directed mutagenesis of a critical cysteine residue

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cDNA coding for human placental thromboxane synthase (EC 5.3.99.5) was amplified by PCR from a human placental cDNA library and sequenced. This cDNA and a shorter cDNA isolated from a human lung cDNA library with a deletion of 163 bp near the 3' end were expressed in *Spodoptera frugiperda* (Sf9) insect cells using a baculovirus expression system. The cDNA from human placenta was expressed as an active enzyme (60 kDa) with a specific activity higher than those reported from other cell types, whereas the shorter cDNA was expressed in an inactive form (52 kDa). The active recombinant enzyme appeared to be unglycosylated as the molecular mass and the enzyme

activity were not altered in the presence of tunicamycin. Site-directed mutagenesis was performed to convert a cysteine at position 480 in thromboxane synthase to a serine. This cysteine is found to be highly conserved in related cytochrome *P*-450 enzymes. The mutant enzyme was found to be inactive, although Western blot, immunoprecipitation and SDS/PAGE analysis indicated that the mutant enzyme was expressed at a level comparable with the wild-type enzyme. These results suggest that Cys-480 is essential for the enzyme catalytic activity and that the short-form cDNA may be a non-functional transcript.

INTRODUCTION

Thromboxane A₂ (TXA₂) is a potent vasoconstrictor and platelet aggregation inducer. It is synthesized primarily in platelets and monocytes/macrophages, but is also produced in various tissues including placenta [1–4]. Its synthesis from prostaglandin H₂ (PGH₂) is catalysed by thromboxane synthase. Two separate products, 12-hydroxyheptadecatrienoic acid and malondialdehyde, are concurrently produced from the same substrate [4]. The enzyme has been purified to homogeneity from porcine lung [4] and human platelets [5], and found to be a monomeric 53–58 kDa haemoprotein. On the basis of spectral properties, the enzyme was proposed to be related to cytochrome *P*-450 [5]. Monoclonal as well as polyclonal antibodies have been produced from respective enzyme [6,7]. Western-blot studies indicated variable molecular masses from different tissues and the presence of two immunoreactive proteins in certain cells [6]. The enzyme undergoes a suicide inactivation during catalysis [8]. Although the biosynthesis of TXA₂ has been extensively studied in various biological systems, little is known about the structure–function relationship and the detailed catalytic mechanism of the enzyme. This is primarily due to the difficulty of obtaining a sufficient amount of the purified active enzyme.

A full-length cDNA for thromboxane synthase was recently cloned from human platelets [9] and human lung [10]. In fact, two different forms of cDNA were noted in the human lung cDNA library. The long form encoded a 60 kDa protein which was essentially identical with that found in human platelets. The short form encoded a 52 kDa protein which differed by the deletion of a 163 bp sequence near the 3' end of the translated region [10]. It was decided to see if both forms could be expressed as a biologically active enzyme.

In the present study, we have cloned a long-form cDNA for thromboxane synthase from a human placental cDNA library by

PCR amplification and have successfully expressed it as active enzyme in insect cells using a baculovirus expression system. The amount of enzyme expressed appeared to be higher than that found in other cell types. We have also attempted to express the short-form cDNA from human lung in the same system. However, little activity could be detected even though the protein was expressed in a similar amount with the predicted size. The same expression system was also used to carry out a site-directed mutagenesis study in which a highly conserved Cys-480 in the cytochrome *P*-450 gene family was changed to a serine. The mutated recombinant protein exhibited no enzyme activity, indicating that Cys-480 is essential for enzyme catalysis.

EXPERIMENTAL

Materials

TA cloning kit, Grace's liquid and powdered insect medium, transfer vector pVL 1392/1393, *Spodoptera frugiperda* (Sf9) insect cells and *Autographa californica* multiply-enveloped nuclear polyhedrosis virus (AcMNPV) were obtained from Invitrogen. Fetal bovine serum was from GIBCO-BRL, and Taq polymerase was from Perkin–Elmer Cetus. Sequenase version 2.0 DNA sequencing kit and other DNA-modifying enzymes were from United States Biochemical Corp. Immobilon-P (polyvinylidene fluoride) (PVDF) membrane was obtained from the Millipore Corp. The anti-mouse IgG–alkaline phosphatase conjugate was from Zymed Laboratories. PGH₂ was obtained from Cayman Chemical Co. Human placental λ gt11 cDNA library was kindly provided by Dr. Jose Millan (La Jolla Cancer Research Foundation). Human lung λ gt11 cDNA library was obtained from Clontech. Monoclonal and polyclonal antibodies to porcine lung thromboxane synthase were prepared as described previously [6]. Oligonucleotide primers for PCR and sequencing were synthesized by the Macromolecular Structure and Analysis

Abbreviations used: TX, thromboxane; PG, prostaglandin; Sf9 cells, *Spodoptera frugiperda* cells; AcMNPV, *Autographa californica* multiply-enveloped nuclear polyhedrosis virus; PVDF, poly(vinylidene difluoride).

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Facility of the University of Kentucky. The Altered Sites *In Vitro* Mutagenesis Kit was obtained from Promega. Other biochemicals were obtained from various commercial sources.

Human thromboxane synthase cDNA cloning and subcloning

Human placental thromboxane synthase cDNA was amplified from a human placental λ gt11 cDNA library by PCR with synthetic oligonucleotide primers using the published human platelet thromboxane synthase cDNA sequence [9]. The sequence of the forward primer is 5'-GTCTCAGAGGAATGATGGAAGCC-3' and that of the reverse primer 5'-TGTGTCA-GCGGGATACGATCTTG-3'. PCR amplification was carried out in a Perkin-Elmer thermal cycler for 32 cycles. The first two cycles consisted of 3 min at 94 °C, 2 min at 42 °C and 3 min at 72 °C. The next 30 cycles consisted of 1 min at 94 °C, 2 min at 55 °C and 3 min at 72 °C. The PCR product was cloned into pCR1000 TA cloning vector. The DNA sequence was determined by Sanger's dideoxynucleotide method [11]. After sequence confirmation, the cDNA was subcloned into transfer vector pVL1392 at *NotI* and *XbaI* to generate pVL1392-TX. A short form of human lung thromboxane synthase cDNA was obtained by screening a human lung λ gt11 cDNA library with 32 P-labelled 5' end fragment (635 bp) of the long-form cDNA. PCR was used to place an *XmaI* site at the 5' end and an *XbaI* site at the 3' end of the short-form cDNA coding sequence. This PCR product was then cloned into pVL1393 at *XmaI* and *XbaI* sites to create pVL1393-TXS. The entire insert in this plasmid was sequenced to ensure no misincorporation during PCR.

Cell culture

Sf9 cells were grown at 27 °C in Falcon tissue culture flasks or dishes in TNM-FH medium supplemented with 10 % fetal bovine serum as described by Summers and Smith [12].

Site-directed mutagenesis

Site-directed mutagenesis was performed using the Altered Sites *In Vitro* Mutagenesis Kit. *Bam*HI fragment (from nucleotide 621 to 1609) released from pVL1392-TX was subcloned into pSelect I at the *Bam*HI site forming pSelect-TX. Single-stranded DNA produced from this pSelect-TX was used as a template for mutagenesis. The mutagenic primer had the sequence 5'-GGC-CCACGGAGCTCCCTCGGGGTGCGT-3'. This sequence corresponds to nucleotides 1426–1452 of the cDNA except for nucleotide 1439 where C replaced G (underlined in the sequence above). This base change converts the codon for the cysteine at position 480 in the enzyme into a codon for serine. The mutated fragment was inserted back to the *Bam*HI-digested pVL1392-TX. The new recombinant transfer plasmid was designated pVL1392-TXM.

Generation of recombinant baculovirus

Recombinant transfer plasmid purified by CsCl gradient centrifugation (5 μ g) was cotransfected into 2×10^6 Sf9 cells along with 1 μ g of wild-type AcMNPV viral DNA using the calcium phosphate transfection procedure [12]. *In vivo* homologous recombination in Sf9 cells between polyhedrin sequences in the wild-type viral DNA and the recombinant plasmid resulted in the generation of recombinant baculovirus. Recombinant baculoviruses were isolated and purified by several rounds of plaque assay.

Expression of thromboxane synthase cDNA in Sf9 cells

Sf9 cells (1×10^7) were seeded in a 75 cm² tissue culture flask in 20 ml of complete TNM-FH medium. Cells were allowed to attach for 1 h. The medium was removed, and 4 ml of Grace's medium containing recombinant virus at a multiplicity of infection of about 10 was added. After incubation for 1 h at 27 °C, the medium was removed and 20 ml of fresh complete TNM-FH medium was added. The cells were allowed to grow continuously for a certain period of time. Cells were harvested routinely 48 h after infection except for the time-course determination. Cells were collected by centrifugation at 500 *g* (1000 rev./min) for 10 min. The cells were washed with PBS, pH 7.5, and lysed by sonication in 2 ml of 50 mM Tris/HCl, pH 7.5, containing 0.2 mM dithiothreitol and 1 mM phenylmethanesulphonyl fluoride, and stored at –80 °C until use.

Assay of thromboxane synthase activity

Thromboxane synthase activity was assayed as described previously [4]. The synthesis of TXB₂ from PGH₂ was determined by enzyme immunoassay of TXB₂ [13].

SDS/PAGE and immunoblotting

Proteins were fractionated by SDS/10 % PAGE and were transferred electrophoretically to Immobilon-P PVDF membrane as described previously [4]. The membrane was incubated first with mouse monoclonal antibody to porcine lung thromboxane synthase (1:30 diluted ascitic fluid) and then with anti-mouse IgG-alkaline phosphatase conjugate. The enzyme immobilized on the membrane was visualized by adding Nitroblue Tetrazolium (0.2 mg/ml) plus 5-bromo-4-chloro-3-indolyl phosphate (0.1 mg/ml).

Protein determination

Protein concentration was determined by the method of Bradford [14] with BSA as standard.

RESULTS AND DISCUSSION

The 1.6 kb coding region (encoding 534 amino acids) of the long-form cDNA for human placental thromboxane synthase was amplified by PCR from a human placental cDNA library. The cDNA was cloned into pCR 1000 TA cloning vector to form pCR1000-TX and the cDNA sequence was determined. The entire coding sequence was found to be identical with the reported human platelet cDNA sequence [9] except for a single base change in codon 386 (from GGC to AGC). The cDNA was then cut out from pCR1000-TX by *NotI* and *SpeI* and subcloned into transfer vector pVL1392 at *NotI* and *XbaI* sites to create pVL1392-TX. The short-form cDNA from the human lung cDNA library that we isolated by plaque hybridization contains a 1380 bp open reading frame (encoding 460 amino acids). The entire coding sequence is identical with the reported short-form human lung cDNA [10] except for two base changes in codon 195 (from GCC to CGC). The base change is probably not caused by misincorporation during PCR as this change was also seen in two other independent PCR clones. Different individuals and tissues may have some sequence variation without affecting enzyme function. The first 456 amino acids of the short-form thromboxane synthase are identical with the long form, which contains 534 amino acids. The last four amino acids are different from the long form. This appears to be caused by a 163 bp deletion near the 3' end of the coding region in the long-form

Figure 1 Time course of thromboxane synthase activity expressed in AcMNPV-TX-infected Sf9 cells

Sf9 cells grown in monolayers were infected with the wild-type (wt) virus AcMNPV or the recombinant virus AcMNPV-TX and harvested at 24, 48, 72 and 96 h after infection. Portions (50 μ g) of these lysates were assayed for enzyme activity as described in the Experimental section. Each point represents an average of two determinations.

Figure 2 SDS/PAGE analysis of the expressed thromboxane synthase from Sf9 cells

Lysates were from Sf9 cells infected with wild-type or recombinant viruses 48 h after infection. The lysate proteins (75 μ g/lane) were fractionated on an SDS/10% polyacrylamide gel and stained with Coomassie Blue. Each lane represents lysate from different virus-infected Sf9 cells. Lane 1, AcMNPV; lane 2, AcMNPV-TXS; lane 3, AcMNPV-TXM; lane 4, AcMNPV-TX. The arrows indicate the positions of 60 kDa (top) and 52 kDa (bottom) proteins.

cDNA. As there are no convenient restriction sites in the short-form cDNA, the PCR was used to place an *Xma*I site near the 5' end and an *Xba*I site near the 3' end of the cDNA coding sequence. The PCR product was digested with *Xma*I and *Xba*I and subcloned into pVL1393 to form pVL1393-TXS. The insert in this plasmid was resequenced to ensure no misincorporation during the PCR. The construction of the mutant form pVL1392-TXM is described under 'Site-directed mutagenesis' in the Experimental section. Transfer vectors pVL1392 and pVL1393 are the same in all characteristics except for the multiple cloning sites which are in opposite orientations. cDNA cloned in these vectors will produce non-fusion protein when expressed in Sf9

Figure 3 Western-blot analysis of the expressed thromboxane synthase from Sf9 cells

Proteins (200 μ g/lane) in unstained gels were blotted on Immobilon-P PVDF membrane and then detected with monoclonal anti-(porcine lung thromboxane synthase) antibody and anti-mouse IgG-alkaline phosphatase conjugate as described in the Experimental section. Each lane represents lysate from different virus-infected Sf9 cells. Lane 1, AcMNPV; lane 2, AcMNPV-TX; lane 3, AcMNPV-TXM; lane 4, AcMNPV-TXS.

cells. The recombinant baculoviruses were generated by co-transfecting the recombinant transfer plasmids into Sf9 cells along with wild-type AcMNPV viral DNA. The recombinant baculovirus AcMNPV-TX was identified and purified by plaque assay combined with functional enzyme assay. The recombinant baculoviruses AcMNPV-TXM and AcMNPV-TXS were identified and purified by plaque assay combined with PCR analysis.

The time course of thromboxane synthase expression in AcMNPV-TX-infected Sf9 cells was determined as shown in Figure 1. Maximal activity of thromboxane synthase in the lysate occurred 48 h after infection, with a specific activity of 31.1 nmol of TXB₂/min per mg of protein, whereas no enzyme activity could be detected in the lysate of wild-type virus AcMNPV-infected Sf9 cells. The specific activity of crude cell lysate is even higher than that of microsomes prepared from various types of mammalian cells, including platelets [15]. This is mainly due to a relatively high level of expression of the enzyme in Sf9 cells. We have purified the recombinant enzyme and estimated that the enzyme content was about 1% of the total cell proteins. The expressed enzyme has a molecular mass of 60 kDa, as revealed by the presence of an intensified protein band of the indicated molecular mass which is barely found in control cells, as shown in Figure 2 (lane 4 compared with lane 1). The 60 kDa protein from active cells but not from control cells was also specifically recognized by a mouse monoclonal antibody generated against porcine lung thromboxane synthase [6] in a Western-blot analysis, as shown in Figure 3 (lane 2 compared with lane 1).

The expressed active enzyme in the cell lysate was further characterized. The K_m value for PGH₂ calculated from a double-reciprocal plot was 10 μ M, which was comparable with that estimated for the enzyme from porcine lung [4] and human platelets [5]. On the basis of the deduced amino acid sequence, the enzyme has five potential sites (Asn-Xaa-Thr/Ser) for *N*-glycosylation. It is not clear if the enzyme is glycosylated. To examine this issue, the enzyme was expressed in the presence of 2 μ g/ml tunicamycin, a glycosylation inhibitor, under conditions that inhibited fully the glycosylation of other proteins [16]. Neither the enzyme activity nor the molecular mass, as revealed

Table 1 Enzyme activity of expressed thromboxane synthase and the C480S mutant in Sf9 insect cells

Sf9 cells were infected with recombinant and control viruses AcMNPV-TX, AcMNPV-TXS, AcMNPV-TXM and AcMNPV. The thromboxane synthase activities in various Sf9 cell lysates were determined 48 h after infection. Each value represents the average of duplicate assays.

Variant of recombinant viruses	Specific activity (pmol of TXB ₂ /min per mg of protein)	Activity (%)
Long form (AcMNPV-TX)	31 000	100
Short form (AcMNPV-TXS)	34	0.11
C480S mutant (AcMNPV-TXM)	16	0.05
Control (AcMNPV)	0	0

by Western blot, was altered in the presence of the inhibitor (results not shown). Furthermore, endoglycosidase H treatment of the partially purified thromboxane synthase also failed to change the enzyme activity and the molecular size. It is therefore concluded that the enzyme is probably not a glycoprotein.

The short-form thromboxane synthase cDNA isolated from the human lung cDNA library was similarly expressed in the insect cells. As there are no convenient restriction sites in the short-form cDNA for direct cloning into transfer plasmid pVL1392/1393, PCR was used to introduce an *Xma*I site at the 5' end and an *Xba*I site at the 3' end of the coding region. The PCR product was then cloned into pVL1393 to create pVL1393-TXS. The insert in this plasmid was sequenced to ensure no misincorporation during PCR. The recombinant baculovirus AcMNPV-TXS was isolated by plaque assay and the presence of the right size of cDNA insert (1.4 kb, results not shown) was confirmed by PCR analysis. After infection of Sf9 cells with the AcMNPV-TXS virus, very little thromboxane synthase activity could be detected. Table 1 compares the activity of the wild-type enzyme with that of the short-form enzyme. The short-form enzyme (52 kDa) was expressed at a similar level to the wild-type enzyme (60 kDa) as indicated by Western-blot analysis (Figure 3, lane 4 compared with lane 2). Similar findings were also observed by ³⁵S-labelling of the enzyme followed by immunoprecipitation (results not shown). However, less than 1% of the wild-type enzyme activity could be detected for the short-form protein (Table 1). These results indicate that the short-form cDNA encodes a biologically inactive enzyme. The short form could well be an alternative splicing product, as the diverging and converging sequences of both forms are GT and AG which are consensus sequences for an alternative splicing signal. Synthesis of an inactive enzyme may provide a means of regulating the expression of a biologically active enzyme at the level of RNA splicing. The presence of two immunologically reactive bands (60 kDa and 53 kDa) in human lung fibroblast WI-38 [6] is consistent with this interpretation, although proteolytic cleavage is another possibility.

Thromboxane synthase was previously shown to be a membrane-bound haemoprotein related to the cytochrome *P*-450 family [5,17]. The most highly conserved region in the *P*-450 family is the haem-binding site. X-ray crystallography studies in *P*-450cam have revealed that the haem is sandwiched between two nearly parallel α -helices, I and L [18]. The highly conserved cysteine residue near the L helix in the *P*-450 enzyme has been proposed to constitute the fifth ligand of the haem iron [19,20]. Substitution of histidine or tyrosine for this cysteine in rat liver *P*-450d prevents haem incorporation into the mutant protein and

474	G	A	G	P	R	S	C	L	G	V	R	484	TXA synthase
436	G	S	G	P	R	N	C	I	G	M	R	446	Human <i>P</i> -450IIIA4
436	G	S	G	P	R	N	C	I	G	M	R	446	Human <i>P</i> -450HFLA
373	G	H	G	S	H	L	C	L	G	Q	H	383	<i>Pseudomonas putida</i> <i>P</i> -450cam
456	G	W	G	V	R	Q	C	L	G	R	R	466	Human <i>P</i> -450scc
455	G	W	G	V	R	Q	C	V	G	R	R	465	Bovine <i>P</i> -450scc
435	G	L	G	K	R	K	C	I	G	E	T	445	Mouse <i>P</i> -450p1
430	S	T	G	K	R	I	C	L	G	E	G	440	Rat <i>P</i> -450b
422	G	C	G	A	R	V	C	L	G	E	P	432	Human <i>P</i> -450C21
421	G	C	G	A	R	V	C	L	G	E	S	431	Bovine <i>P</i> -450C21
430	S	L	G	K	R	I	C	L	G	E	G	440	Rabbit <i>P</i> -450LM2

Figure 4 Similarity of thromboxane synthase to other cytochrome *P*-450 enzymes

The sequence shown corresponds to residues 474–484 of the thromboxane synthase. Numbers on the left and the right are the corresponding position of each *P*-450 enzyme. The arrow indicates Cys-480 of thromboxane synthase, which is conserved in each of these *P*-450s. Human *P*-450IIIA4 and *P*-450HFLA sequences are from ref. [10]. All other sequences are from ref. [19].

causes the loss of catalytic activity of the enzyme [21]. Comparison of the deduced amino acid sequence of thromboxane synthase with these *P*-450s show that the thromboxane synthase has significant sequence similarity to other *P*-450s in its C-terminal region [10], particularly those domains corresponding to the conserved helices, I and L, and the cysteine pocket near the L helix. Figure 4 compares the amino acid sequence (residues 474–484) of thromboxane synthase with some selected *P*-450 enzymes. A cysteine at position 480 in thromboxane synthase is among the most highly conserved amino acids. It may play an important role in haem binding in thromboxane synthase. Furthermore, the group-specific modifying reagent, *N*-chlorosuccinimide, was shown to completely inactivate porcine lung thromboxane synthase [22]. This suggests that certain cysteine residue(s) might be involved in the catalytic activity of the enzyme. Therefore it was decided to change the codon for this Cys-480 to a codon for serine by site-directed mutagenesis. The C480S mutant thromboxane synthase was expressed in Sf9 cells using the baculovirus expression system under the same conditions as were used to express the wild-type enzyme. Although the mutant protein was expressed at a similar level to the wild-type enzyme, as revealed by the Western-blot analysis (Figure 2, lane 3 compared with lane 4, and Figure 3, lane 3 compared with lane 2), very little thromboxane synthase activity could be detected in the Sf9 cells infected with AcMNPV-TXM (Table 1). These results lead us to conclude that the Cys-480 in thromboxane synthase is essential for catalytic activity. The fact that the short form exhibited little enzyme activity is consistent with the lack of this cysteine residue in the C-terminal region of the protein.

Note added in proof (received 13 August 1993)

While this manuscript was in review, a report related to the expression of thromboxane synthase in insect cells was published by Yokoyama et al. [23].

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REFERENCES

- Needleman, P., Moncada, S., Bunting, S., Vane, J. R., Hamberg, M. and Samuelsson, B. (1976) *Nature* (London) **261**, 558–560

- 2 Yoshimoto, T., Yamamoto, S., Okumura, M. and Hayaishi, O. (1977) *J. Biol. Chem.* **252**, 5871–5874
- 3 Morley, J., Bray, M. A., Joens, R. W., Nugteren, P. H. and Van Dorp, D. A. (1979) *Prostaglandins* **17**, 730–736
- 4 Shen, R. F. and Tai, H. H. (1986) *J. Biol. Chem.* **261**, 11592–11599
- 5 Haurand, M. and Ullrich, V. (1985) *J. Biol. Chem.* **260**, 15059–15067
- 6 Shen, R. F. and Tai, H. H. (1986) *J. Biol. Chem.* **261**, 11585–11591
- 7 Nüsing, R., Wernet, M. P. and Ullrich, V. (1990) *Blood* **76**, 80–85
- 8 Jones, D. A. and Fitzpatrick, F. A. (1990) *J. Biol. Chem.* **265**, 20166–20171
- 9 Yokoyama, C., Miyata, A., Ihara, H., Ullrich, V. and Tanabe, T. (1991) *Biochem. Biophys. Res. Commun.* **178**, 1479–1484
- 10 Ohashi, K., Ruan, K. H., Kulmacz, R. J., Wu, K. K. and Wang, L. H. (1992) *J. Biol. Chem.* **267**, 789–793
- 11 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- 12 Summers, M. D. and Smith, G. E. (1987) *Tex. Agric. Exp. Stn. Bull.* **1555**
- 13 Tai, H. H. (1992) in *Encyclopedia of Pharmaceutical Technology*. Vol. 5 (Swarbrick, J. and Boylan, J. C., eds.), vol. 5, pp. 201–233, Marcel Dekker, New York
- 14 Bradford, M. (1976) *Anal. Biochem.* **72**, 248–253
- 15 Ullrich, V. and Nüsing, R. (1990) *Stroke (Dallas)* **21**, IV-134–IV-138
- 16 Jarvis, D. L. and Summers, M. D. (1989) *Mol. Cell. Biol.* **9**, 214–223
- 17 Nüsing, R., Schneider-Voss, S. and Ullrich, V. (1990) *Arch. Biochem. Biophys.* **280**, 325–330
- 18 Poulos, T. L., Birzel, B. C., Gunselus, I. C., Wagner, G. C. and Kraut, J. (1985) *J. Biol. Chem.* **260**, 16122–16130
- 19 Nelson, D. R. and Strobel, H. W. (1988) *J. Biol. Chem.* **263**, 6038–6056
- 20 Nebert, D. W. and Gonzalez, F. J. (1987) *Annu. Rev. Biochem.* **56**, 945–993
- 21 Shimizu, T., Hivano, K., Takahashi, M. and Hatano, M. (1988) *Biochemistry* **27**, 4138–4141
- 22 Shen, R. F. (1985) Ph.D. thesis, University of Kentucky
- 23 Yokoyama, C., Miyata, A., Suzuki, K., Nishikawa, Y., Yoshimoto, T., Yamamoto, S., Nüsing, R., Ullrich, V. and Tanabe, T. (1993) *FEBS Lett.* **318**, 91–94