In Situ Proteolysis for Crystallization of Membrane Bound Cytochrome P450 17A1 and 17A2 Proteins from Zebrafish

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Fish and human cytochrome P450 (P450) 17A1 catalyze both steroid 17α-hydroxylation and 17α,20-lyase reactions. Fish P450 17A2 catalyzes only 17α-hydroxylation. Both enzymes are microsomal-type P450s, integral membrane proteins that bind to the membrane through their N-terminal hydrophobic segment, the signal anchor sequence. The presence of this N-terminal region renders expression of full-length proteins challenging or impossible. For some proteins, variable truncation of the signal anchor sequence precludes expression or results in poor expression levels. To crystallize P450 17A1 and 17A2 in order to gain insight into their different activities, we used an alternative N-terminal sequence to boost expression together with in situ proteolysis. Key features of our approach to identify crystallizable P450 fragments were the use of an N-terminal leader sequence, a screen composed of 12 proteases to establish optimal cleavage, variations of protease concentration in combination with an SDS-PAGE assay, and analysis of the resulting fragments using Edman sequencing. Described in this unit are protocols for vector preparation, expression, purification, and in situ proteolytic crystallization of two membrane-bound P450 proteins. © 2016 by John Wiley & Sons, Inc.

Keywords: in situ proteolysis • crystallization • membrane protein

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INTRODUCTION

Cytochrome P450 enzymes (P450s) play crucial roles in the metabolism of endogenous and exogenous compounds (Ortiz de Montellano, 2005). Microsomal P450s also participate in the detoxification of xenobiotics and the synthesis of steroid hormones (Guengerich, 2005; Miller and Auchus, 2011). The hydrophobic N-terminal leader sequence of microsomal P450s includes a membrane-spanning helix that targets them to the endoplasmic reticulum where the catalytic domain of about 460 amino acids is lodged on the cytoplasmic side of the membrane (Johnson and Stout, 2013). Hydrophobic patches on helix A and the connector between the F/F′ and G helices provide additional anchoring points on the membrane surface (Fig. 29.16.1). Like many other membrane proteins, membrane-bound P450s remain challenging targets for recombinant expression and crystallization. Thus, *Escherichia coli* (*E. coli*) continues to be the most commonly used organism for overexpression of recombinant proteins, including P450s (Guengerich and Martin, 2006); however, it may not be capable of providing the folding machinery and specific lipid environment for production of a membrane protein in milligram quantities that are needed for biophysical and structural investigations. Even with pure protein

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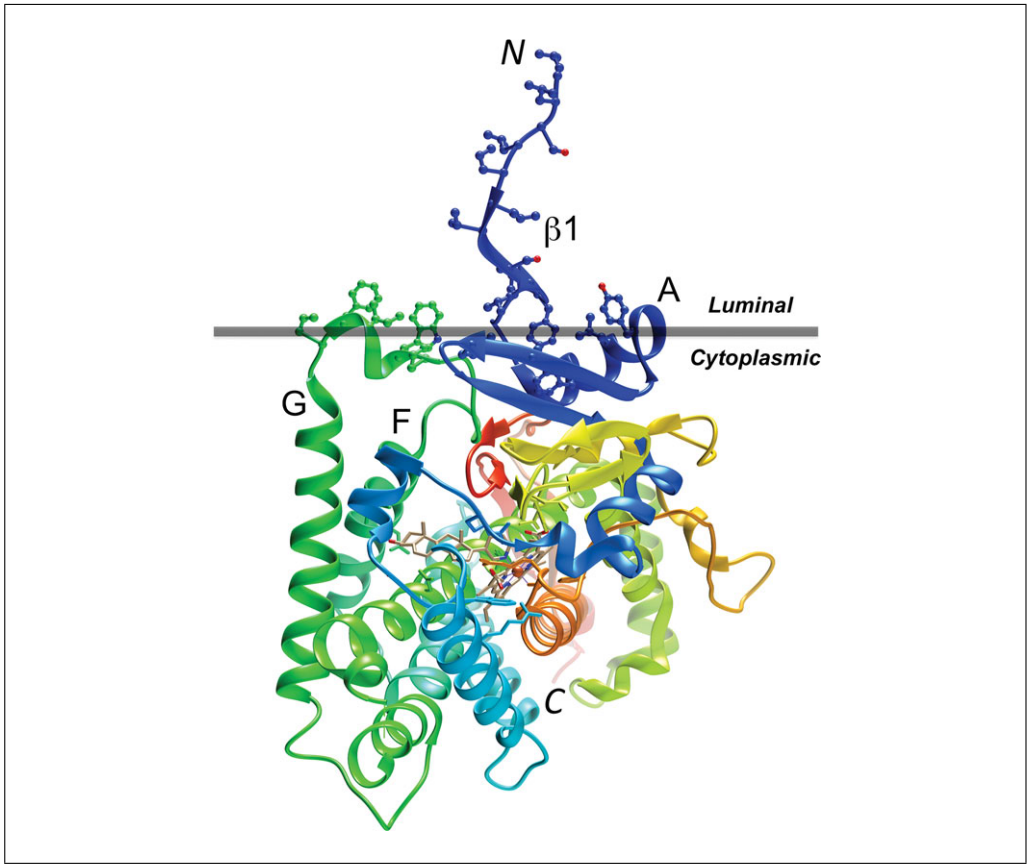

Figure 29.16.1 Tertiary structure diagram of human P450 17A1 (PBD ID code 3RUK; *http://www.rcsb.org)* with the ribbon colored in the rainbow spectrum, from N-terminus (blue) to C-terminus (red). Amino acid side chains in hydrophobic regions of the protein associated with the membrane (horizontal gray bar) are depicted in ball and stick mode. The P450 enzyme is located on the cytoplasmic side of the bilayer (Monk et al., 2014). The image was generated with the program UCSF Chimera (Pettersen et al., 2004).

available, the presence of flexible hydrophobic N- and/or C-terminal tails in P450s can frequently hamper crystallization efforts, and then requires a time-consuming search for alternative protein constructs that can be expressed in high yields and are suitable for production of diffraction-quality crystals. Our experiments suggest that truncation of the N-terminal membrane anchor sequence will not affect P450 function and, combined with addition of a soluble N-terminal linker, will increase protein expression yields and solubility. Moreover, co-expression of chaperon EL/ES assists in protein folding. Compared to this approach, the use of eukaryotic expression systems such as Sf9 insect cells is expensive, time consuming and often suboptimal in terms of yields. However, P450 enzymes expressed in *E. coli* lack post-translational modifications such as phosphorylation, glycosylation, ubiquitination or nitration that have been observed in various eukaryotic P450 subfamilies (Lamb and Waterman, 2013).

To date, 57 human P450s have been identified and among these at least 14 serve important roles in steroid metabolism (Guengerich, 2005; Miller and Auchus, 2011). For example, human P450 17A1 is primarily expressed in adrenal and testicular tissues and catalyzes two-step oxidation reactions of progesterone and pregnenolone. In the first step, 17α-hydroxylation of these substrates results in 17α-hydroxy-progesterone and 17α-hydroxy-pregnenolone, respectively. The 17α,20-lyase reaction in the second step subsequently produces androstenedione and dehydroepiandrosterone, respectively. Interestingly, the reactions catalyzed by P450 17A1 are dependent on the cellular location of the enzyme. Thus, in the gonads 17A1 converts the 21-carbon steroid to the 19-carbon androgen in a

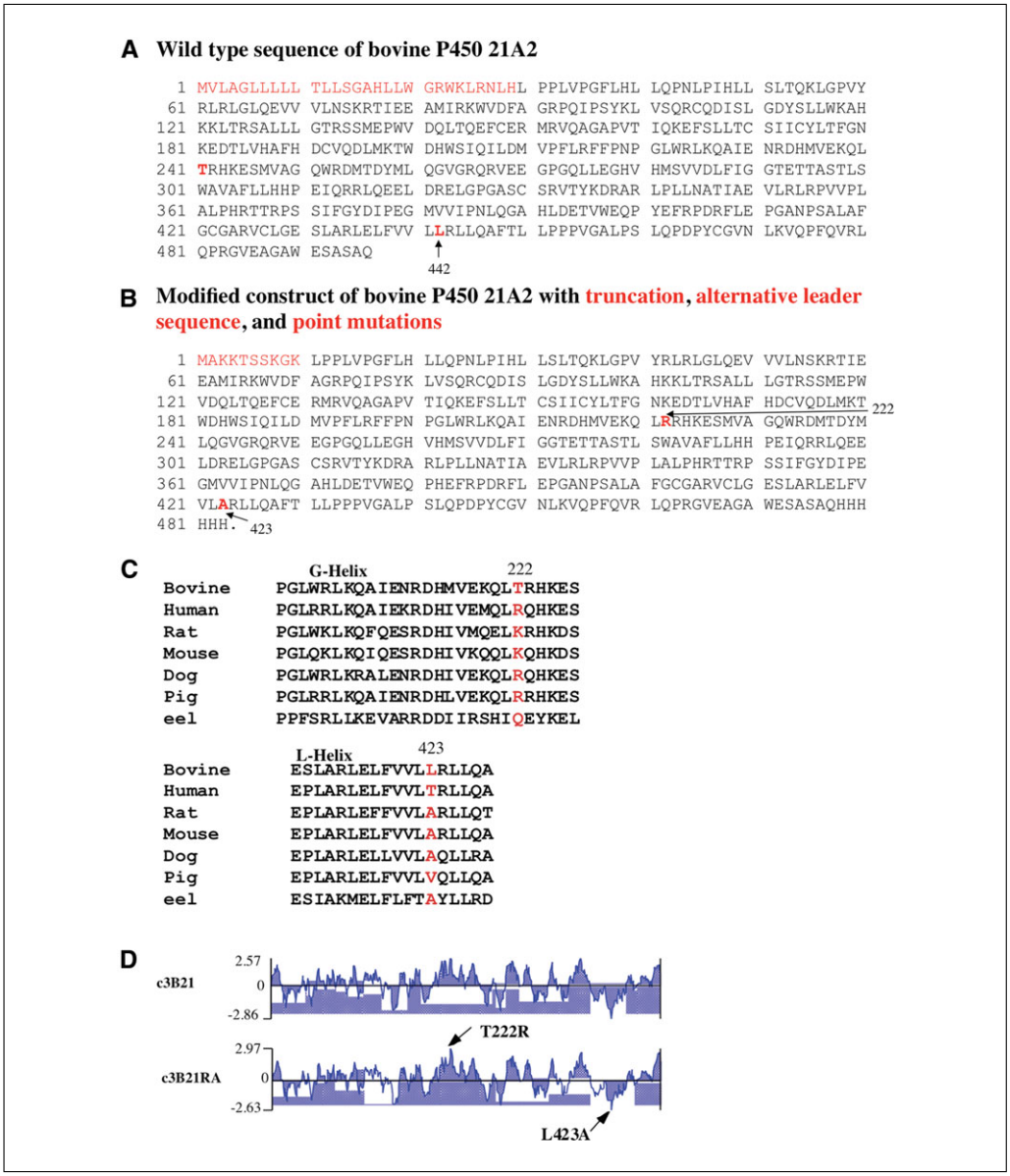

Figure 29.16.2 Generation of alternative P450 constructs for improving expression, solubility, stability, and crystal formation, using bovine P450 21A2 as an example (Zhao et al., 2012). (**A**) Wild-type sequence of bovine P450 21A2. (**B**) A soluble fragment was obtained by replacing the hydrophobic N-terminal 29 amino acids in bovine P450 21A2 by the shorter MAKKTSSKGK leader sequence (from the N-terminus of P450 2C3). Residues that were mutated individually are highlighted in red in the wild type (Thr-241 and Leu-442) and alternative protein constructs (Arg-222 and Ala-423, respectively). (**C**) Identification of candidate surface residues for point mutation (T241R and L442A) by aligning the sequences of P450 21A2 from selected eukaryotic species, combined with (**D**) hydrophilicity analyses of wild type (with alternative leader; c3B21) and mutated protein sequences (c3B21RA). The alternative protein construct shown in panel b was used for determining the crystal structure of bovine P450 21A2 (Zhao et al., 2012).

3-step hydroxylation reaction. Conversely, in the adrenal cortex the enzyme stops after the initial hydroxylation reaction (Lamb and Waterman, 2013).

For catalytic activity, P450 enzymes require electrons that are provided by reduced nucleotides NADPH and NADH and redox partner proteins that shuttle the electrons to the iron center: Fe-containing ferredoxin (Fdx) and flavin adenine dinucleotide (FAD)-containing ferredoxin reductase (FDR) (prokaryotic P450s); flavin mononucleotide (FMN)/FAD-containing NADPH cytochrome P450 oxidoreductase (CPR) (eukaryotic

P450s); and adrenodoxin (ADX) and adrenodoxin reductase (ADR) (mitochondrial P450s) (Lamb and Waterman, 2013). In some cases, cytochrome *b*5 can provide the second electron (Johnson and Stout, 2013).

Prostate cancer cells proliferate in response to androgen steroids, and inhibition of the 17α,20-lyase reaction only is a new strategy to prevent androgen synthesis and treat lethal metastatic, castration-resistant prostate cancer without interfering with mineralocorticoid and glucocorticoid synthesis. The drug abiraterone that inhibits both enzymatic functions of human P450 17A1 is used in the clinic for treatment of metastatic castration-resistant prostate cancer (DeVore and Scott, 2012). Teleost fish feature two P450 17 A enzymes; like human P450 17A1, fish P450 17A1 catalyzes both steroid 17α-hydroxylation and 17α,20-lyase reactions. However, P450 17A2 only catalyzes the 17α-hydroxylation. We analyzed the substrate binding properties, enzymatic behavior and three-dimensional structures of P450 17A1 and 17A2 from zebrafish in order to gain insight into the biochemical basis of the 17α,20-lyase reaction, and to potentially identify differences in the active sites between the enzymes that could explain the absence of the lyase reaction in 17A2 (Pallan, et al., 2015a). Both enzymes are microsomal-type P450s, integral membrane proteins that bind to the membrane through their N-terminal hydrophobic segment. Extensive attempts to crystallize the two proteins failed, despite testing a variety of constructs with portions of the N-terminal regions truncated and/or alternative leader sequences (Pallan, et al., 2015a), mutation of N-terminal amino acids, and screening a large number of crystallization conditions.

The present protocol describes an approach that allowed us to successfully grow diffraction-quality crystals for both P450 17A1 and 17A2 from zebrafish. In the first step, we produced the two membrane proteins in *E. coli* both in good quality and quantity by truncating the N-terminal hydrophobic sequence, and adding a leader sequence, in addition to co-expressing the GroEL/ES chaperonin to assist P450 protein expression and folding (Fig. 29.16.2A,B). Secondly, we screened twelve different proteases in regard to their cleavage patterns with P450 17A1 and 17A2, by varying protease concentration in combination with a gel electrophoretic assay and analyzing the resulting fragments using Edman sequencing. Compared to the time-consuming preparation of discrete expression systems for proteins with systematically shortened N-terminal regions, in situ proteolysis allows for potentially rapid identification of crystallizable fragments. Thirdly, the 17A1 and 17A2 proteins featuring an N-terminal leader sequence conducive for expression (Richardson et al., 1995), in conjunction with the optimal protease for generating N-terminally truncated proteins, were used for in situ proteolysis in the crystallization and subsequent structure determination.

STRATEGIC PLANNING

Experimental Design

Expression system

E. coli remains a commonly used organism for expression of membrane proteins as it is relatively inexpensive and numerous proteins can be produced in recombinant form easily and efficiently. BL21 cells and derivatives thereof offer multiple choices such as different cell culture temperatures (14°C to 37°C) to optimize expression of a wide range of target proteins, including membrane or toxic proteins and those encoded by rare codons. To increase target protein expression and augment folding, a GroEL/ES chaperonin expression vector is co-transformed into *E. coli* BL21 (DE3) cells.

Protein construct

Eukaryotic membrane-bound P450s have a hydrophobic N-terminal anchor region that serves to attach the protein to the membrane. N-Terminal truncation of P450s can affect

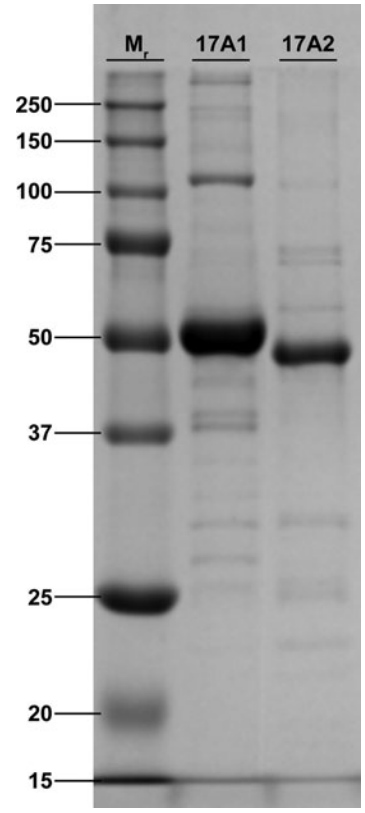

Figure 29.16.3 SDS-PAGE of expressed and purified P450 17A1 and 17A2 proteins from zebrafish.

both activity (e.g., by limiting proximity between the P450 catalytic domain and the reductase that are both anchored to the membrane) and expression levels. Thus, in our experience protein constructs with truncated N-terminal portions fail to express or the expression levels are poor. Replacing the missing hydrophobic region by a soluble linker can sometimes rescue protein expression in *E. coli*. In our case we introduced the P450 2C3 N-terminal sequence that is known to improve expression (Richardson et al., 1995; Zhao et al., 2012) (Fig. 29.16.2A,B). Alternative leader sequences include a histidine tag at the N-terminal end that enables both increased protein expression and protein purification by Ni-NTA agarose affinity chromatography. However, we introduced a hexa-histidine tag encoded by 18 nucleotides at the C-terminal end. We relied on the pET17 expression vector for producing both the P450 17A1 and 17A2 proteins of zebrafish. Other pET expression vectors or pET-derived vectors include pAT, pBG and pSV. With some vectors, it is possible to introduce a specific protease cleavage site between protein and tag or the leader to help cleave off the latter. If, after truncating the N- or C-terminal hydrophobic tails, and replacing the former with a leader sequence and co-expression of GroES/EL chaperonin, protein expression and/or solubility could not be improved, we used the Protean software package (*https://www.dnastar.com/t-protean.aspx)* to analyze the protein sequence and, based on a sequence alignment, picked hydrophobic amino acid(s) that map to the surface of the protein and mutated them to less hydrophobic or hydrophilic amino acid(s) as identified in the same protein subfamily or in other species in the alignment (Fig. 29.16.2). In an alternative mutation experiment, we produced the P450 17A1 C57R mutant in order to test whether trypsin in combination with this single arginine mutation would promote crystal growth, because in situ proteolysis with trypsin and cleavage at an N-terminal arginine had previously produced diffraction-quality crystals of P450 17A2 (Pallan et al., 2015a).

The zebrafish P450 17A1 and 17A2 enzymes were expressed at levels of between 500 and 1100 nmole/liter of culture (Fig. 29.16.3) and used for both biochemical experiments and crystallization (Pallan et al., 2015a).

Detergent screen

Not every membrane-bound P450 protein requires the use of detergent for crystallization. Thus, we relied on 5-cyclohexyl-1-penthyl-β-D-maltoside to crystallize bovine P450 21A2 (Zhao et al., 2012), but no detergent was used to produce crystals of human P450 21A2 (Pallan et al., 2015b). In cases where the protein cannot be concentrated to 20 mg/ml at 50 mM potassium phosphate, pH 7.0-7.4, 5% to 10% glycerol, 50-100 mM NaCl, and 1 mM DTT, like with zebrafish P450 17A1 and 17A2, a detergent that helps solubilize the membrane protein needs to be identified. A good starting point is the use of a screening kit such as the Detergent Screen HT offered by Hampton Research.

In situ proteolysis and crystallization

Following prescreening of 12 proteases, optimal cleavage conditions and protease concentrations were determined. For the crystallization experiments, we used a mosquito nanodrop setting robot (Molecular Dimensions) in combination with 96-well plates. For each well in the plate, two droplets containing the protein with or without addition of the protease were set up and nearly 300 different crystallization conditions screened. After several days, crystals were observed only in drops that were treated with the respective proteases. Initial diffraction tests indicated that these diffracted to resolutions of between 2.8 and 3 Å. Edman sequencing revealed that the N-terminal residue of P450 17A2 in the crystals was Ser-52, consistent with trypsin cleaving C-terminal to Arg-51 and in accord with the first residue visible in the electron density maps being Leu-54. In the case of P450 17A1 treatment with subtilisin and analysis of the proteolytic products with SDS-PAGE indicated a mixture of fragments. Edman sequencing of the major product placed the N-terminus at Gly-37. However, the crystal contained a shorter fragment as indicated by Ser-83 being the first residue visible in the electron density maps.

BASIC PROTOCOL

VECTOR PREPARATION, EXPRESSION, PURIFICATION, AND IN SITU PROTEOLYTIC CRYSTALLIZATION OF MEMBRANE-BOUND P450 PROTEINS

This protocol provides instructions for the generation of expression plasmids for P450 proteins with alternate N-terminal sequences, their expression in *E. coli*, purification of the protein by nickel affinity, ion exchange, and size exclusion chromatography, detergent screening, in situ proteolytic crystallization experiments, and analysis of the protein in crystals using N-terminal Edman sequencing. The experimenter may also consult existing protocols on the preparation of plasmids, expression in *E. coli*, protein purification and crystallization.

Materials

Zebrafish
Plasmid vectors: pET *17b* expression vectors (Novagen)
Qiagen One-Step RT-PCR kit
E. coli DH5 cells for cloning (Vanderbilt University Core Lab)
BL21 (DE3) Gold (or plysS) for overexpressing protein (Invitrogen)
LB agar plates
Antibiotics: e.g., Kanamycin sulfate (Research Products International, cat. no. K22000-5.0), Ampicillin sodium salt (Research Products International, cat. no. A40040-5.0)
LB broth (see recipe)

δ-Aminolevulinic acid hydrochloride (ALA; CHEM-IMPEX International, cat. no. 01433)
L-Arabinose (Gold Bio Technology, CAS No. 87-72-9)
IPTG (Research Products International, cat. no. I56000-5.0)
Tris
TES buffer
Lysozyme
Buffer A (see recipe for buffers for chromatography)
Ice
Ni-NTA Agarose resin (Qiagen, cat. no. 30250)
Imidazole (Sigma, cat. no. I2399-500 G)
Buffer B (see recipe for buffers for chromatography)
Liquid nitrogen
Sepharose
Deionized water
Buffer C (see recipe)
Sodium chloride (NaCl)
CMC detergent (see recipe)
C12E9 Polyoxyethylene(9)dodecyl Ether • Thesit • Polydocanol • α-Dodecyl-w-Hydroxy-Poly(oxy-1,2-Ethanediyl) (Anatrace, cat. no. Apo-12950X1ml)
Buffer D (see recipe)
For SDS-PAGE: mini-protean TGX precast 4% to 20% (w/v) Tris-glycine gel, (BIO-RAD, cat. no 601-1105)
Coomassie Brilliant Blue R 250 (MP Biomedical, cat. no. 04821616)
Pregnenolone (Steraloids), 17α-OH pregnenolone (Steraloids), and Abiraterone (Selleckchem)
Ethanol
Dimethyl sulfoxide (DMSO)
Phenylmethanesulfonyl fluoride (PMSF; Sigma, cat. no. P7626-25 G)
Laemmli Sample buffer for SDS-PAGE (Bio-Rad, cat. no. 161-0737)
SDS gel stain (see recipe)
SDS gel detain solution (see recipe)
Crystallization screens:
  Index kit (Hampton Research, cat. no. HR2- 144)
  Crystal screen (Hampton Research, cat. no. HR2-110)
  Crystal screen 2 (Hampton Research, cat. no. HR2-112)
  PEG/Ion screen (Hampton Research, cat. no. HR2-126)
Trypsin
Seed bead (Hampton Research, cat. no. 2-320)
Methanol (Fisher Scientific, cat. no. A433P-4)
3-Cyclohexylamino-1-propanesulfonic acid (CAPS buffer; see recipe)
K_2HPO_4 and KH_2PO_4 (Research Products International, cat. no. 41300-500 and 41200-500, respectively)
Detergent Screen HT (Hampton Research, cat. no. HR2-406)
Proti-Ace Kit (Hampton Research, cat. no. HR2-429)
Proti-Ace 2 Kit (Hampton Research, cat. no. HR2-432)

37°C incubator
250-ml and 2.8-liter flasks
Incubator shaker
Spectrophotometer (Shimadzu Scientific, cat. no. UV-2401PC)
Beckman GS6 KR centrifuge (Beckman Instrument, cat. no. 362134)
50-ml tubes

Sonicator
Protean software package (*https://www.dnastar.com/t-protean.aspx*)
1.5-ml microcentrifuge tubes
CrystalQuick 96-well Greiner 609191 (Hampton Research, cat. no. HR3-192)
Microwave oven
Mosquito crystal liquid handler (TTP labtech, cat. no. Mosquito)
Rock Imager (Formulatrix cat. no., rock imager 1000)
Vortex mixer
Invitrolon PVDF filter paper sandwich, 0.45-μm pore size (Invitrogen, cat. no. LC2005)
MRC 2 well crystallization plates (Hampton Research, cat no. HR3-082)
Amicon ultra centrifugal filters, 30 K (Merck Millipore, cat. no. UFC 903024)
Q sepharose fast flow (GE Healthcare, cat. no. 17-0510-01)
SP sepharose fast flow (GE Healthcare, cat. no. 17-0720-01)
Size-exclusion columns
Superdex 75 10/30 GL (GE Healthcare, cat. no. 17-5174-01)
ClearSeal film (Hampton Research, cat. no. HR4-521)
Ultracentrifuge Optima L-80 (Beckman Instruments, cat. no. 355884)
FPLC (GE Amersham Biosciences, cat. no. 11001479 AKTAbasic UPC10 incl. Frac-920)

Generate the modified expression vector

1. Design and order primers for PCR amplification of the P450 17A1 and P450 17A2 DNAs (extracted from zebrafish). For more information regarding primer design, please see Kuslich et al. (2008). The 3′-primer contains the codons of the *Nde*I site, MAKKTSSKGK, and codons for five amino acids of the truncated N-terminal region of the target proteins. The 5′-primer contains the codons of the *Hind*III site, stop codon, the region encoding six histidines and codons for five C-terminal amino acids of the target proteins. Use RNA extracted from zebrafish as template with the Qiagen One Step RT-PCR kit and follow the manufacturer's instructions to amplify the genes. Alternatively, order the N-terminal truncated gene with the *Nde*I site and MAKKTSSKSK in the protein's N-terminal region and the *Hind*III site, stop codon and six histidines in the protein's C-terminal region.

2. Clone the modified genes into the pET17b vector using the *Nde*I and *Hind*III sites and confirm them by sequencing: In the zebrafish P450 17A1 open reading frame, the region encoding the N-terminal transmembrane helix (residues 1–26) was replaced by DNA coding for MAKKTSSKGK (P450 2C3 N-terminal region), and the 3′-end was extended by 18 nucleotides encoding six histidines. For zebrafish P450 17A2, the region encoding the N-terminal transmembrane helix (residues 1–25) was replaced by DNA coding for MAKKTSSKGK (P450 2C3 N-terminal region) and the 3′-end was extended by 18 nucleotides encoding six histidines. Both modified cDNAs were inserted into the pET17b vector.

   If no colonies are obtained, see Table 29.16.1 for troubleshooting suggestions. For more details regarding molecular cloning, please see Green and Sambrook (2012).

Express the protein in E. coli

3. Co-transform *E. coli* BL21 (DE3)Gold or BL21(DE)3 PlysS cells by heat shock according to the manufacturer's protocol with the vectors containing the target gene and pGro12 ES/EL gene. Plate the transformed cells onto LB agar plates with the appropriate antibiotic selection. Grow overnight at 37°C.

4. Pick several colonies and inoculate 20 ml LB broth with the appropriate antibiotics in a 250-ml flask. Culture cells in an incubator/shaker overnight at 37°C and 225 rpm.

Table 29.16.1 Critical Parameters and Troubleshooting

Step	Problem	Possible reason	Solution
1-2	No colonies	Wrong antibiotic or the concentration of the antibiotic is too high. Competent cells are not viable. Insufficient competent cells or plasmids. Culturing time at 37°C is too short.	Switch to the correct antibiotic. Lower the concentration of the antibiotic. Use fresh competent cells. Use a larger amount of competent cells or plasmids. Culture the plate at 37°C for up to 20 hr.
3-6	Very low or no protein expression	Competent cells are not viable or glycerol stock was used for an overnight culture. Used wrong medium for overnight culture Overnight culture at 37°C is too long Insufficient expression time. Expression temperature is incorrect. Target protein is toxic. The target protein gene codons are not optimal for *E.coli*. The protein is expressed in inclusion bodies.	Use healthy competent cells, and make a fresh transformation. Use LB medium. Culture at 37°C for less than 17 hr. Determine the best time course. Determine the best temperature course. Switch to competent cells that tolerate toxic proteins. Switch to BL21Codonplus expression cells. Switch to expression competent cell line that allows expression of protein at low temperature, such as Arctic Expression competent cells. Add an N-terminal or C-terminal soluble fragment. Mutate hydrophobic amino acid(s) on the surface of the protein. See Experimental Design.
7-12	The protein does not bind to Ni resin.	DTT or EDTA concentration is too high. Affinity tag is sequestered by detergent micelle or is buried inside the protein. Too much imidazole in the lysis buffer.	Lower the concentration. Lower the detergent concentration or add the linker between the protein and the affinity tag. Lower imidazole concentration to 2-5 mM.
	The protein from Ni resin is heavily contaminated.	The proteins from the host cell bind to Ni resin non-specifically.	Add to 5-10 mM imidazole and 100 mM glycine to lysis buffer. Increase imidazole to 20-50 mM in wash buffer.
13-17	Protein does not bind to ion exchange column.	The protein carries the same charge as the resin. Ionic strength of eluent is too high. Protein is denatured or has precipitated.	Check the pI and the charge of protein in the buffer. Lower the ionic strength. Keep or add detergent to the protein solution. After loading, immediately wash with buffer containing 50-100 mM NaCl.
30-33	No protein bands on either the SDS gel or PVDF.	Not enough protein loaded onto the gel. Protein is smaller than 15 kDa which passes PDVF.	Load more protein. Remove SDS from blotting buffer or increase MeOH concentration.
	Protein band cannot be trans-ferred to PVDF membrane.	Protein is bigger than 80 kDa.	Add 0.1-0.2% SDS or omit MeOH from blotting buffer.

5. Using 2.5 ml of the overnight growth culture started in step 4, dilute 1:100 in 250 ml of TB medium, prepare 6 individual 2.8-liter flasks with the 250 ml of diluted overnight growth culture, and add the appropriate antibiotics. Grow the cultures in an incubator shaker at 37°C and using 175 to 225 rpm. After 3.5 to 4 hr (OD600 reaches 1.0-1.2), add 1 mM ALA to boost heme production and 2 mg/ml arabinose to induce chaperones. After another hour, induce target protein expression by adding IPTG to a final concentration of 1 mM. Continue to grow the cultures at 26°C and at 100-120 rpm. for 36 hr.

   Achieving high expression of target protein is one of the critical steps for obtaining enough purified protein to screen crystallization conditions. For overnight cultures at 37°C, the optimal incubation time is less than 17 hr. If more time is needed, it is preferable to culture at 32°C. Freshly transformed clones usually result in more optimal expression. The culturing time after IPTG induction is dependent on the individual protein; it may be necessary to determine the best time course for a particular target protein.

   We normally use 1 mM ALA to help E. coli produce more heme and the ALA concentration can be increased up to 2 mM. For example, 2 mM ALA affords better expression of a fusion protein that consists of two proteins that both require heme. Regarding the arabinose concentration: if the experiment shows a large amount of chaperone expression—in any case more than needed by the target protein—decrease the arabinose concentration or induce chaperone and target protein expression at the same time and decrease the culture temperature.

6. Transfer the culture to centrifuge tubes and harvest the cells by centrifuging for 25 min at 2000 × *g*, 4°C. Discard the supernatant and resuspend the pellet in 80 to 100 ml of 100 mM Tris·Cl, pH 8.0, or TES buffer, pH 8.0. Add lysozyme to 1 mg/ml, keep the sample at 4°C and stir for 25 min. Collect cells by centrifuging for 25 min at 2000 × *g*, 4°C. Discard the supernatant and store overnight cells at −80°C.

   The cells can be frozen at this point and stored at −80°C. Overnight storage is recommended as freezing and thawing will promote cells lysis, but tolerance for storage should be determined for each target protein. Lysozyme treatment is also important for cell lysis.

   If expression levels are low or expression fails, see Table 29.16.1 for troubleshooting suggestions.

Purify target protein

All steps should be carried out on ice or at 4°C and all buffers should be kept at 4°C. Since our target proteins are colored, no FPLC is needed. The following procedure is based on using our own packed columns and the gravity method except for the size exclusion column.

Nickel affinity purification

7. Add 200 to 300 ml of buffer A to thaw and lyse the cells, then transfer the sample to 50-ml tubes and sonicate three times for 30 sec each time on ice for each tube to break cells and DNA. Centrifuge the sample for 40 to 50 min in an Ultracentrifuge Optima L-80 at 111,000 × *g*, 4°C. Collect the supernatant.

8. Prepare the appropriate amount (typically 15-20 ml) of Ni-NTA agarose resin according to the manufacturer's instructions. Equilibrate the column with 50 ml buffer A.

9. Load the supernatant from step 7 onto the column, collect and save the flow-through containing the unbound material. Measure the CO spectrum (400-500 nM wavelength range) for P450 protein, and take a 10- to 20-μl sample of the flow-through for analysis by SDS-PAGE and store up to 3 days at −20°C.

   The flow-through should contain no or only a small amount of the target protein.

10. Wash the column with buffer A until the signal of the eluent at 280 nm returns nearly to baseline and then wash with 50 to 100 ml of buffer A containing 10 to 20 mM imidazole. Check the flow-through P450 CO spectrum and take a 10-μl sample of the wash out for SDS-PAGE analysis and store up to three days at −20°C. Wash the column with 50 to 100 ml of buffer A and 50 ml buffer B until the flow-through OD280 nm returns to nearly baseline.

11. Elute the protein with buffer B containing 200 to 300 mM imidazole and collect the peak(s). Take a 2- to 10-μl sample for analysis by SDS-PAGE and store up to three days at −20°C. Flash freeze sample in liquid nitrogen and transfer to−80°C.

12. *Optional:* The column can be washed with up to 50 mM imidazole wash buffer, dependent on how tightly the target protein binds to Ni-NTA or how large an amount of target protein is bound to the column. Some target protein may be sacrificed in exchange for obtaining purer protein.

    If problems are encountered with the Ni-NTA chromatography, see Table 29.16.1 for troubleshooting suggestions.

Ion-exchange chromatography

13. Before ion-exchange chromatography, determine the size, pI and the charges etc. of the target protein using the Protean software.

14. Make "Q" and "SP" columns by adding 6 to 10 ml Q and SP sepharose, respectively, into the columns, and washing them with 50 ml of deionized water and then 20 ml buffer B.

15. Dilute the eluent from the Ni-NTA column 1:1 with buffer C, load onto the "Q" sepharose fast flow column with a flow rate of 1 ml/min and then load the sample which passes the "Q" onto the "SP" sepharose fast flow column. Wash with 50 ml buffer C containing 50 mM NaCl and then with 50 ml buffer C containing 100 mM NaCl until OD 280 nm returns to base line.

16. Elute the sample with buffer C containing 250 to 300 mM NaCl. Take a 1- to 5-μl sample of the elution for SDS-PAGE analysis and store up to 3 days at −20°C.

17. If a size-exclusion column is needed for further purification, using an Amicon ultra centrifugal filter (30K), concentrate the protein sample to 1 to 2 ml. Flash freeze the sample in liquid nitrogen and transfer to −80°C.

    Use freshly made protein for crystallization as soon as possible. The tolerance for storage should be determined for each target protein.

    Passing the target protein through the same charge (as protein; positive/negative) column is important; proteins that carry opposite charge can bind to the column, while others that are nearly neutral in the buffer have the opportunity to bind the ion exchange column they first encounter.

    If the protein does not bind to ion-exchange column, see Table 29.16.1 for troubleshooting suggestions.

Perform detergent screen

18. To produce detergent-free protein, detergents can be eliminated during column purification by reducing their concentration stepwise during washing and simultaneously increasing the NaCl concentration from 50 mM to elution concentration. This can be done while washing the SP column (see step 15) and eluting the protein without detergent.

    Try to avoid concentrating the membrane protein. If it has to be done at all, monitor carefully to determine whether there is precipitation while concentrating the protein.

19. Prepare 10× critical micelle concentration (CMC; differs for individual detergents) detergent stocks. Add 10× CMC detergent to the protein sample (2 to 10 mg/ml) for a final concentration of 1× CMC and place the sample at 20°C. Check if any precipitation occurs and analyze the P450 CO spectrum each day for 7 days with a detergent-free protein control. Select the sample with the highest ratio of P450 to P420 peak. Alternatively, perform a detergent screen using commercial screening kits (detergent screen HT from Hampton Research). Detergent C12E9 was chosen for zebrafish P450 17A1 and 17A2 crystallization.

The authors typically scan the sample between 400 and 500 nm. We prefer proteins that have no peak at 420 nm or a ratio between the P450 and P420 peaks that is no lower than 5:1. Usually we add 30% glycerol to the sample buffer to protect the protein from being damaged by the sodium dithionite reducing agent when we collect the P450 CO spectra.

Size-exclusion chromatography

20. Using an FPLC instrument, equilibrate the Superdex 75 10/30 GL column with buffer D, inject 0.5 ml of sample, and run it with buffer D at a flow rate of 0.5 ml/min. Collect 0.5- to 1-ml fractions of the eluent while monitoring at 280 nm. To determine which peak on the chromatogram contains the target protein, check the P450 peak of the fractions collected or run them on a 4% to 20% (w/v) tris-glycine gel or a native gel and stain with Coomassie (Green and Sambrook, 2012) (Fig. 29.16.3). If only a single peak is obtained and the protein is sufficiently pure for crystallization after the ion-exchange column, no size-exclusion column step is needed.

Prepare P450 substrates and inhibitor complexes for crystallization

21. Measure the concentration of target protein; in a 1:2-10 molar ratio (1:10 for abiraterone inhibitor and 1:2–1:5 for substrates), add either 100 mM progesterone dissolved in ethanol or 10 mM abiraterone dissolved in DMSO, mix well and remove any aggregated protein or particulate matter by centrifuging using a 1.5-ml tube in a microcentrifuge for 10 min at 15,700 × *g*, 4°C.

22. Concentrate the protein-substrate or protein-inhibitor complexes to 30 mg/ml using an Amicon ultra centrifugal filter, 30K. Aliquot the protein to the volume needed for individual crystallization experiments, in our case 22 µl/plate (200 nl × 96 wells; MC2 well crystallization plate). Flash freeze in liquid nitrogen and transfer to −80°C.

In situ proteolysis for crystallization of zebrafish P450 17A1 and 17A2 proteins

23. Prepare 1 mg/ml protease stock solution using the Proti-Ace and Proti-Ace 2 Kits (Hampton Research), as per the manufacturer's suggestion. Prescreen the target protein-to-protease ratio to determine conditions that yield the largest, most stable domain that will be used for subsequent crystallization by mixing protease and target protein at 1:100, 1:1000, and 1:2000 ratios (w/w), and incubating the mixtures at room temperature for 30 min, before adding PMSF to a 1 mM final concentration.

CAUTION: *PMSF is corrosive; avoid inhalation and contact with skin and eyes.*

24. Add same volume of Lämmli sample buffer and boil for 2 min, load on a 4% to 20% Mini-PROTEAN TGX Precast Gel, run the gel at 30 to 40 mA until the blue dye migrates to the bottom of the gel. Stain the gel with 10 ml of SDS gel stain solution by heating in a microwave oven for 15 sec and keeping at room temperature for another 2 min. Destain the gel with SDS gel destain solution until the background is clear.

A 1:1000 protease to target protein ratio was found to be optimal in our case for the crystallization experiments (Figs 29.16.4 and 29.16.5).

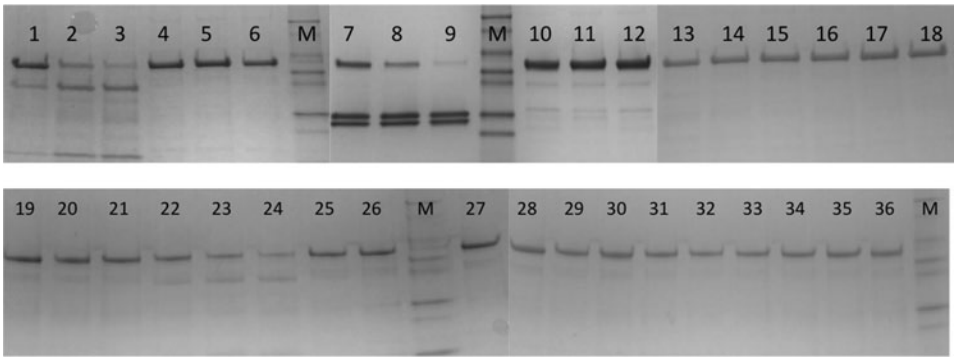

Figure 29.16.4 SDS-PAGE (4% to 20%) gel depicting zebrafish P450 17A1 cleavage with 12 proteases, using various protein to protease ratios: Subtilisin, lane 1 1:2000, lane 2 1:1000, lane 3 1:100; papain, lane 4 1:2000, lane 5 1:1000, lane 6 1:100; trypsin, lane 7 1:2000, lane 8 1:1000, lane 9 1:100; α-chymotrypsin, lane 10 1:2000, lane 11:1000, lane 12 1:100; endoproteinase Glu-C, lane 13 1:2000, lane 14 1:1000, lane 15 1:100; elastase, lane 16 1:2000, lane 17 1:1000, lane 18 1:100; pepsin: lane 19 1:2000, lane 20 1:1000, lane 21 1:100; protease K, lane 22 1:2000, lane 23 1:1000, lane 24 1:1000; thermolysin, lane 25 1:2000, lane 26 1:1000, lane 27 1:100; actimase, lane 28 1:2000, lane 29 1:1000, lane 30 1:100; bromelain, lane 31 1:2000, lane 32 1:1000, lane 33 1:100; clostripain, lane 34 1:2000, lane 35 1:1000, and lane 36 1:100. M represents a molecular marker lane.

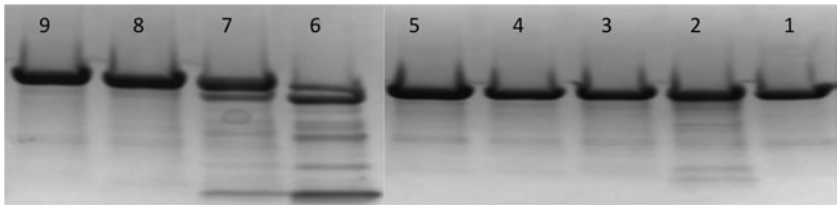

Figure 29.16.5 SDS-PAGE (4% to 20%) gel depicting zebrafish P450 17A2 cleavage with trypsin or α-chymotrypsin using various protein to protease ratios: α-chymotrypsin, lane 1 1:0, lane 2 1:100, lane 3 1:1000, lane 4 1:10000, lane 5 1:100000; and trypsin, lane 6 1:100, lane 7 1:1000, lane 8 1:10000, and lane 9 1:100000.

If the protease has no specific cleavage site within the protein sequence, the ratios among protein fragments observed in the SDS-PAGE gel may not be representative of those formed in the crystallization drop. Ratios vary with temperature, screening reagent condition and the time of exposure of the protein to the particular protease. The size of the protein in crystals formed in different screen conditions may be different.

Crystallization experiments

25. At room temperature, dispense 60 μl of a commercially available crystallization screen (conditions 1–96) into reservoirs 1–96 of an MRC 2 well crystallization plate.

26. Prior to setting up crystallization droplets, add subtilisin (trypsin; 1 mg/ml stock concentration for both proteases) to zebrafish P450 17A1 (zP450 17A2) inhibitor (or substrate) complex with the protease-to-P450s ratio (w/w) being 1:1000.

    The authors usually use a protein concentration of 20 to 30 mg/ml to screen crystals.

27. Remove 10 μl of the mixture and incubate at room temperature for 30 min, then add PMSF to a 1 mM final concentration, boil for 2 min and store at −20°C for subsequent N-terminal sequence analysis (step 30).

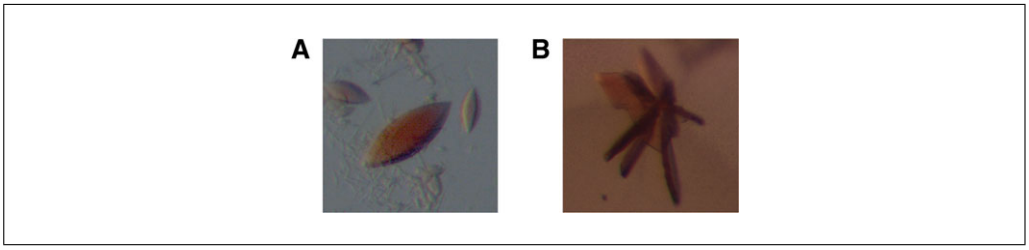

Figure 29.16.6 Crystals of zebrafish P450 (**A**) 17A2 and (**B**) 17A1, obtained by in situ proteolysis using trypsin and subtilisin, respectively. The modified zebrafish P450 17A1 and P450 17A2 proteins were expressed using plasmids based on the pET17b vector. The protease to target protein ratio for in situ proteolytic crystallization was 1:1000 and the maximum size of crystals was ca. 0.25 mm. Images taken using a Leica MZ28 stereomicroscope.

28. Using a Mosquito crystal liquid handler, set sitting drops, two drops per well, one by mixing 200 nl mother liquor with 200 nl protein substrate or inhibitor complex, the other by mixing 200 nl of protein substrate or inhibitor complex and protease mixture with 200 nl of a given screening condition, and cover with ClearSeal film. Incubate the 96-well plates in a Rock Imager at 21°C and capture images on days 1, 3, 5, 7, 14, 21, 30 and once a month after day 30.

    Crystals of P450 17A2 with abiraterone or progesterone appeared within 3 to 4 days. Their size was around 0.2-0.4 mm (Fig. 29.16.6A). Crystals of P450 17A1 with abiraterone appeared within 5 to 7 days and, as the crystals were smaller than 0.05 mm, their size was increased by a round of microseeding.

29. Microseeding is carried out as follows:

    a. Pick 3 to 5 crystals (if they can be scooped up with a nylon loop).
    b. Place the crystals in a 1.5-ml microcentrifuge tube with 20 to 30 µl of reservoir solution and a seed bead, or use 5 to 10 µl of reservoir solution to wash the well that contains microcrystals, and put the mixture into a 1.5-ml microcentrifuge tube with 40 µl of reservoir solution and a bead.
    c. Vortex the tube for 1 to 2 min. Dilute the mixture with reservoir solution at ratios of 1:400, 1:800, 1:1600, and 1:3200, and use these diluted mixtures to seed drops by mixing 200 nl of protein inhibitor complex and protease mixture with 200 nl of the seed solution.

        Crystals of P450 17A1:abiraterone complex (0.15 to 0.3 mm size) appeared within 3 to 10 days (Fig. 29.16.6B).

N-terminal Edman sequencing

30. Using buffer C, dilute 50-fold the samples that were treated with protease before making crystallization setups (step 23), then add an equal volume of SDS-gel sample buffer, boil for 2 min, and load 20 µl to each lane of a mini-protean TGX precast 12% (w/v) tris-glycine gel. Run the gel at 30 to 40 mA until the blue dye reaches the bottom of the gel.

31. Soak PVDF in 10 ml methanol and then equilibrate with 10 ml CAPS buffer. Rinse the gel with 50 ml deionized water and briefly keep in CAPS buffer.

32. Assemble gel PVDF sandwiches as follows: gel holder-fiber pad-filter paper-gel-PVDF membrane-filter paper-fiber pad-gel holder. Put the sandwiches in the electrophoresis tank filled with CAPS buffer with the gel on the cathode side. Run at 30 V/100 mM, for 16 hr (overnight) at 4°C or in a cold room.

33. Stain the PVDF blot with 10 ml of Coomassie blue R-250 stain solution until the protein bands appear and using SDS gel destain solution, destain the blot until the

background is light. Thoroughly rinse the blot with deionized water. Cut out the target bands with a razor blade and dry them at room temperature.

The cut-out bands are now ready for N-terminal Edman sequencing. If no bands are visible on the gel or the membrane, see Table 29.16.1 for troubleshooting suggestions.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps.

ALA stock solution

Dissolve δ-Aminolevulinic acid hydrochloride (ALA) into water to 1 M
Store up to three months at −20°C

Buffers for chromatography

If possible, always try to use freshly prepared buffer solutions. However, buffers can be used on consecutive days and stored at 4°C for up to one week.

Buffer A: cell lysis and Ni-NTA column equilibration and washing. Mix 50 mM potassium phosphate (K_2HPO_4 and KH_2PO_4; Research Products International, cat. nos. 41300-500 and 41200-500, respectively) (pH 7.4), 300 to 500 mM NaCl, 0.1 mM Dithiothreitol (DTT; Research Products International, cat. no. D11000-5.0), 0.1 mM EDTA (Research Products International, cat. no. D11000-5.0), 20% (v/v) glycerol, 1%(v/v) Tween-20 (Anatrace, cat. no. T1003), 1% (w/v) sodium cholate (Sigma, cat. no. C1254).

Buffer B: Ni-NTA wash buffer. Mix 50 mM potassium phosphate (pH 7.4), 0.1 mM DTT, 0.1 mM EDTA, 20% (v/v) glycerol, 1% (v/v) Tween-20, 1% (w/v) sodium cholate.

Buffer C: Q and S sepharose fast flow column. Mix 50 mM potassium phosphate (pH 7.4), 0.1 mM DTT, 0.1 mM EDTA, and 0.005% (v/v) C12E9.

Buffer D: Size-exclusion chromatography.

CAPS buffer: PVDF electroblotting buffer

Dissolve 2.21 g of 3-Cyclohexylamino-1-propanesulfonic acid (CAPS; Research Products International, cat no. c30060) in 500 ml deionized H_2O
Add 100 ml methanol
Add 10 ml of 10% SDS
Adjust volume to 1 liter using deionized H_2O
Adjust pH to 11 with NaOH
Store up to one week at 4°C

The SDS concentration used in CAPS buffer depends on the size of the target protein.

LB agar plates

Prepare 1 liter of LB broth (see recipe) and add 15 g of Bacto Agar (Becton, Dickinson and Company, cat. no. 214010) before autoclaving, cool to 50°C after autoclaving and add appropriate antibiotic. Dispense into sterile 10-cm diameter petri dishes and allow to set at room temperature. Store plates up to one month at 4°C.

LB broth

Add 25 g of LB Broth Miller (EMD, cat. no. 1.10258.5007) to 1 liter of water and autoclave for 25 min. Store at room temperature (25°C) until use.

PMSF stock

Dissolve phenylmethanesulfonyl fluoride (PMSF) in DMSF to 0.1 M and store up to six months at −20°C

SDS gel destain

Mix 120 ml methanol, 120 ml of glacial acetic acid (Fisher Scientific, cat. no. A38-212), and 460 ml water. Store up to three months at room temperature.

CAUTION: *Glacial acetic acid is corrosive, avoid inhalation and exposure to skin and eyes.*

SDS gel stain

Make 0.2% (w/v) stock solution as manufacturer recommends: 1 tablet (PhastGel Blue R pre-measured tablets; Sigma-Aldrich B4921) in 80 ml of ultra pure water, and add 120 ml of methanol. Store up to three weeks at 4°C.

SDS-PAGE gel running buffer

Dissolve 1 g of SDS, 3 g of Tris base (Research Products International, cat. no. T60040) and 14.4 g of glycine in 600 ml of water. Add water to a final volume of 1 liter. Store up to one month at room temperature.

SDS protein-loading dye, 2×

Mix 950 µl of Laemmli sample buffer (Bio-Rad, cat. no. 161-0737) with 50 µl of 2-mercaptoethanol before use.

TB broth

Add 47 g Terrific Broth (Research Products International Corp., cat. no. T15000-500.0) and 4 ml glycerol to 1 liter of water, autoclave for 25 min. Store up to one week at room temperature.

TES buffer

For lysozyme treatment of cells: 100 mM Tris·Cl, pH 8.0, 1 mM EDTA, 500 mM sucrose

Store up to two months at 4°C

COMMENTARY

Background Information

An initial report on the use of in situ proteolysis with either trypsin or chymotrypsin involving 55 bacterial and 14 human proteins that had proved resistant to crystallization found a 15% success rate, including the steps from pure protein to structure determination (Dong et al., 2007). With an expanded set of 270 soluble proteins and testing six proteases (trypsin, chymotrypsin, elastin, papain, subtilisin and V8 protease), the success rate was ~13% (Wernimont and Edwards, 2009). Both studies were primarily concerned with increasing the number of crystallizable protein fragments as part of a structural genomics effort. Several other preliminary protein crystallization notes and publications involving structure determination of proteins or domains thereof reported the use of in situ proteolysis (Gaur et al., 2004; Johnson et al., 2006; Taneja et al., 2006; Forsgren et al., 2009; Little et al., 2012; Civril and Hopfner, 2014; Kobayashi et al., 2014). At least in two cases, the importance of proteolysis for growing diffraction-quality crystals was a serendipitous discovery in that the solution was infected with a fungus that secreted the protease (Mandel et al., 2006; Bai et al., 2007). Instead of screening crystallization conditions directly by in situ proteolysis, it may be advisable to apply limited proteolysis of a protein prior to crystallization in order to test the suitability of the protease (e.g., Forsgren et al. (2009)]. It is possible that in situ proteolysis will produce a fragment enabling successful crystallization of a protein, but subsequent interference of the protease with crystal growth may hamper crystal quality. In such a case, the cleavage site can be established using SDS-PAGE and mass spectrometry or other means of sequencing and the construct then reengineered for optimal crystal quality (Little et al., 2012). Although only one or at best a few proteases were tested in the context of crystallization in most cases, the use of in situ proteolysis with a larger number of proteins in structural genomics projects (Dong et al., 2007; Wernimont and Edwards, 2009) points to some proteases that seem to

be particularly useful for paving the road to diffraction-quality crystals. Thus, trypsin, chymotrypsin and subtilisin appear to have been used successfully more often than other proteases. However, while one may want to try these three first, it remains a good idea to test as many proteases as possible to increase the likelihood of obtaining diffraction-quality crystals.

Although in situ proteolysis to promote crystallization has been used in quite a few studies, no experimental protocol describing the detailed individual steps, including the screening of proteases, establishing optimal concentrations for their use in situ, and establishing the cleavage site, has been published to date. The present protocol seeks to fill that void and provide biochemists and structural biologists with guidelines based on the successful crystallization of two membrane-bound P450 enzymes using in situ proteolysis that can be applied to any protein or protein domain resisting crystallization. In our efforts to crystallize the P450 17A1 and 17A2 enzymes from zebrafish in complex with substrate and/or inhibitor, we screened an increased set of proteases (12) compared with previous reports. Moreover, we combined efforts to optimize expression using alterative leader sequences at the N-terminal end with the identification of fragments by in situ proteolysis that yield to crystallization. To establish optimal conditions for in situ proteolytic crystallization, we tested a wide range of concentrations and time periods. In addition, we used a combination of SDS-PAGE and Edman sequencing to rapidly and reliably identify fragment size and cleavage sites.

Although the expanded set of proteases tested undoubtedly constitutes an advantage over published accounts of the use of in situ proteolysis to promote crystallization and relying on one or a few proteases (it is possible that the authors may not have disclosed how many were tested or not mentioned those that proved to be unhelpful), it is likely that inclusion of even more proteases may prove beneficial for the crystallization success rate. We relied on commercially available protease kits (Proti Ace and Proti-Ace 2; Hampton Research; *https://hamptonresearch.com).* However, many more proteases than those included in the kits can be purchased individually from a variety of vendors.

In a further extension of the in situ proteolysis approach to macromolecular crystallization, we tested whether mutation to arginine of a residue in the N-terminal region of P450 17A1 in combination with trypsin would promote crystallization. Trypsin cleaves C-terminal to Arg or Lys (Olsen et al, 2004), and we had established that trypsin-catalyzed cleavage at the corresponding arginine in the N-terminal region of P450 17A2 promoted crystallization. Although the mutation in the N-terminal sequence of P450 17A1 ultimately proved not to be conducive to the production of diffraction-quality crystals of this enzyme, variations in the length or amino acid composition of flexible N- and C-terminal tails or internal loop regions could turn out to be fruitful for promoting crystal growth.

Critical Parameters and Troubleshooting

Advice regarding critical parameters and troubleshooting is summarized in Table 29.16.1.

Anticipated Results

The in situ proteolytic crystallization approach is expected to be beneficial for proteins with hydrophobic (including membrane-spanning domains such as those found in microsomal cytochrome P450 enzymes) and/or flexible N-terminal regions in general that are targeted for analysis by single crystal X-ray diffraction or small angle X-ray scattering (SAXS). For the latter application, it is important to identify protein domains or fragments that lack flexible N- or C-terminal regions that interfere with the conformational analysis of the proteins and their complexes in solution. Unlike the P450 17A1 and 17A2 proteins from zebrafish for which in situ proteolysis proved key to successful crystal structure determination (Pallan et al., 2015b), the human P450 17A1 protein could be crystallized by shortening the N-terminal leader sequence and the first residue visualized in the electron density was Leu-31 (DeVore and Scott, 2012). In the crystal lattice hydrophobic N-termini of human P450 17A1 mediate dimer formation, whereby β1 strands are part of an extended β-sheet (Fig. 29.16.7). Dimer formation by human P450 17A1 persists in solution, but unlike for crystal lattice formation, where associations among neighboring molecules are a prerequisite, oligomerization hampers solution studies such as SAXS and complicates conformational analysis of proteins and their complexes (Putnam et al., 2007). Conversely, the N-terminally truncated forms of zebrafish P450 17A1 and 17A2 do not dimerize in solution, rendering them suitable for scattering experiments in principle. Beyond the use for

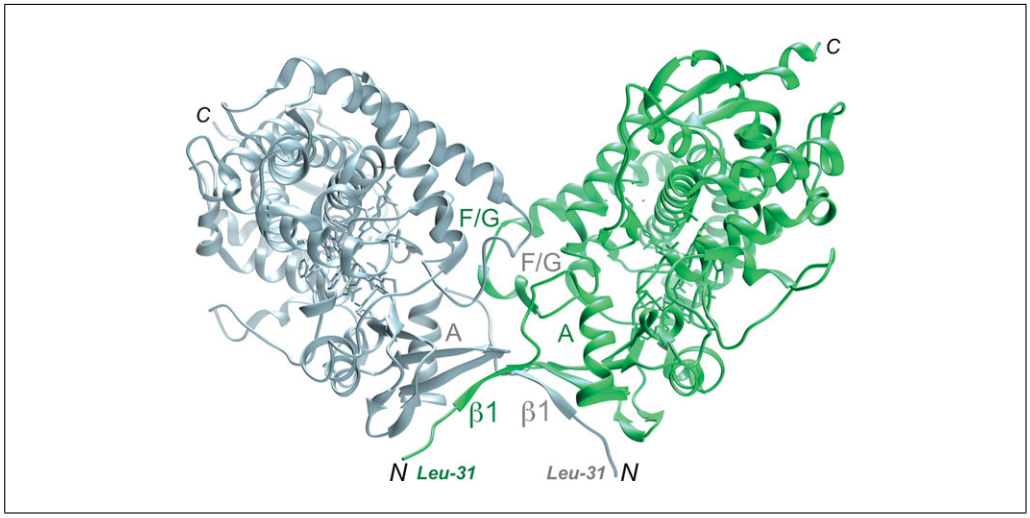

Figure 29.16.7 Dimerization of catalytic domains under formation of an antiparallel β-sheet, mediated by N-terminal hydrophobic tails in the crystal structure of human P450 17A1 in complex with abiraterone (DeVore and Scott, 2012).

structural or biophysical applications, purified proteins that have undergone proteolytic cleavage can also be employed in functional studies and the technique is therefore of interest to structural biologists, biophysicists and enzyme chemists.

Time Considerations

Generating the expression vector: 1 to 2 weeks

Expressing the target protein: 4 to 5 days

Purifying the target protein: 2 to 3 days (using homemade columns; 1 to 2 days with prepacked columns)

Nickel affinity purification: 1 day

Ion-exchange chromatography: 0.5-1 day

Detergent screen: 7 days

Size-exclusion chromatography: 2 hr

Preparations for crystallization experiments: 2 to 5 hr

In situ proteolysis: 1 day

Protein crystallization: 3 to 15 days

N-terminal Edman sequencing: 1-3 days.

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