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

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Path and human cytochrome P450 (P450s) carry out a wide range of metabolic functions. Membrane-bound P450s are part of the endoplasmic reticulum (ER) membrane and play critical roles in the metabolism of a wide range of xenobiotics, including drugs and environmental pollutants. In the ER, P450s are embedded in the lipid bilayer, and their distribution is primarily determined by the lipid phase. The lipid phase affects the stability, activity, and catalytic efficiency of the P450s. In this study, we explored the feasibility of using in situ proteolysis to crystallize membrane-bound P450s. We used a combination of techniques, including cryo-electron microscopy and x-ray crystallography, to visualize the structural changes in the P450s during proteolysis. Our results showed that in situ proteolysis significantly improved the yield and quality of crystals, allowing for the identification of new binding sites and the elucidation of the mechanism of action of the P450s. Overall, our study provides new insights into the mechanisms of membrane-bound P450s and highlights the potential of in situ proteolysis as a powerful tool for the crystallization of membrane proteins.
Basic Protocol

Materials

Zebrafish
Plasmid vectors: pET-17b expression vectors (Novagen)
Qiagen One-Step RT-PCR Kit
E. coli DH5α cells for cloning (Vanderbilt University Core Lab)
BL21 (DE3) Gold strain 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. A40000-5-0)
LB broth (base recipe)
6-L-threomannose acid hydrochloride (A&A/Chem-ImpeX International, cat. no. 01433)
L-Arabinose (Gold Bio Technology, CAS No. 97-72-9)
PTG (Research Products International, cat. no. 165000-5-0)
Tris
TES buffer
LiSO4/PMF
Buffer A (see recipe for buffers for chromatography)
Ice
Ni-NTA Agarose resin (Qiagen, cat. no. 308250)
imidazole (Sigma, cat. no. I395-500 G)
Buffer B (see recipe for buffers for chromatography)
Liquid nitrogen
Sephadex
Diazomethane
Buffer C (see recipe)
Sodium chloride (NaCl)
CMC detergent (see recipe)
Reagents and Solutions

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

**ALA stock solution**
Dissolve 8-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 lysate and Ni-NTA column equilibration and washing. Mix 50 mM potassium phosphate (K2HPO4 and KH2PO4; Research Products International, cat. no. 41300-500 and 41200-500, respectively) (pH 7.4), 300 to 500 mM NaCl, 0.1 mM Dithiothreitol (DTT; Research Products International, cat. no. D11000-50), 0.1 mM EDTA (Research Products International, cat. no. D1 1000-5-0), 20% (v/v) glycerol, 1% (v/v) Tween-20 (Analyticon, cat. no. T1063), 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) C1259.

- Buffer D: Size-exclusion chromatography.

**CAPS buffer: PVDF electrophoresis buffer**
Dissolve 2.21 g of 3-Cyclohexylamino-1-propanesulfonic acid (CAPS; Research Products International, cat. no. c0060) in 500 ml deionized H2O
Add 100 ml methanol
Add 10 ml of 10% SDS
Adjust volume to 1 liter using deionized H2O
Adjust pH to 11 with NaOH
Store at room temperature.
Figures

Introduction

Cytochrome P450 enzymes (P450s) play crucial roles in the metabolism of endogenous and exogenous compounds (Cirri et al., 2019). Mesosomal P450s also participate in the detoxification of xenobiotics and the synthesis of steroid hormones (Guengerich, 2000a; Miller and Auchus, 2011). The hydrophobic N-terminal leader sequence of mesosomal 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-I and G helices provide additional anchoring points on the membrane surface (Fig. 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, 2003); 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 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

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Information

Metrics, Details, Keywords, Publication History

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