Refolding and Reconstitution of CYP101 (only with modest success)

Sample: 50 mg purified P450_{cam} in 5 ml L-buffer.

Procedures:

Aipoprotein formation
1. Remove ionic solute: dialysis the protein sample against aqueous 100 µM camphor solution to remove ions (2 × 12 hr). (Note: buffer exchange using spin column may also work).

2. Heme extraction: combine the protein sample (5 ml in 100 µM camphor) with 5 ml histidine solution (0.2 mM, pH 8.0). Stir under argon for 10 min. Add ~120 µl concentrated HCl, and the pH should be 2.5 (verify using the pH meter). Stir under argon atmosphere and on ice for 30 min, and then add 15 ml ice-cold argon-purged iso-butanol with vertex to extract heme. Spin down at 10K rpm using JA-20 rotor for 15 min, and get rid of the colored upper layer.

3. Remove dissolved ketone: the aqueous phase (straw-colored) protein solution was then dialyzed against 1 liter 0.01% (w/v) degassed and argon-purged NaHCO_{3} solution inside the anaerobic chamber (3 × 12 hr). Apo-P450 will precipitate. (Note: Use a dialysis tubing with extra volume since protein solution expanded substantially during this process).

4. Solublize Apo-P450: Transfer the dialysis tubing containing Apo-P450 precipitant into 500 ml 0.1 M histidine, pH 8.0, 40% glycerol solution inside the anaerobic chamber. Keep dialysis for 8 hrs. The protein solution will be solublized and at the same time dehydrated to ~5 ml.

5. Incubate with DTT: Transfer the protein solution (~5 ml) into a glass vial inside the anaerobic chamber. Add 8 mg solid DTT (final concentration 10 mM) and incubate with stir under argon atmosphere at 25ºC for 1 hr.

6. Remove excess thiol via dialysis: Apo-P450 was finally dialyzed against 500 ml histidine (0.1 M, pH 8.0), 20% glycerol solution inside the anaerobic chamber (3 × 24 hr) to remove excess thiol prior to heme reconstitution.

Heme reconstitution:

1. Transfer the solubilized apo-P450 from the dialysis tubing into a sealable glass vial (20 ml) inside the anaerobic chamber or under argon atmosphere. Bring up the volume to 10 ml by adding appropriate amount of histidine (0.1 M, pH 8.0), 20% glycerol solution (the concentration of the apo-P450 is ~ 0.1 mM). Under argon atmosphere, add 32 mg solid DTT (final concentration 20 mM) and 500 µl camphor-saturated H_{2}O (5% v/v).

2. Dissolve ~1 mg hemin (Sigma # H5533-1G, minimum 80%) in 0.1 ml 0.1 N KOH, then dilute with 0.9 ml H_{2}O. Add 0.8 ml hemin solution drop-wise to the apo-P450 solution with stir under argon.

3. Seal the vial and continue the reconstitution under argon atmosphere for about 45 hrs. During the process, the reconstitution is monitored spectrophotometrically: withdraw 10 µl aliquot of sample using syringe at different time intervals, dilute with L-buffer to 1 ml, reduce with Na_{2}S_{2}O_{4} and flush with CO (ferrous CO form), and measure the peak (or derivative) at 446 nm.
4. Terminate the reconstitution when $A_{446}$ does not change. In my case, 80% P450 formed (absorb at 446 nm) compared to 20% P420 (absorb at ~420 nm). (At the beginning of reconstitution, 0% P450).

5. Concentrate the protein solution and remove the excess free heme by passing the sample through a P-2 column (1.5 $\times$ 20 cm) equilibrated with degassed 50 mM KPi, 0.5 mM camphor.

6. Protein effluent was purified on a HiPrep-DEAE 16/10 fast flow (Amersham) FPLC column developed with a linear 50-250 mM KCl gradient (120 ml total volume) at 4 ml/min flow rate. The final product was almost excluded from P420 (99% P450).

**Problem with current procedure**: low yield. Only ~5% of P450 recovered.

(The reconstitution process can be found in Lingyun Rui Notebook #2, page 22-29)

**Reference**