Structure biology of membrane proteins

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Overall research goals:

The overall goal of the proposed research is to understand the membrane-associated active processes catalyzed by an alkane □-hydroxylase (AlkB) from eubacterium *Pseudomonase oleovorans*. AlkB performs oxygenation of unactivated hydrocarbons found in crude oils. The enzymatic reaction involves energy-demanding steps in the membrane with the uses of structurally unknown metal active sites featuring a diiron [FeFe] center. At present, a critical barrier to understanding the membrane-associated reaction mechanism is the lack of structural information. The structural biology efforts have been challenged by technical difficulties commonly encountered in crystallization and structural determination of membrane proteins. The specific aims of the current budget cycle are to crystalize AlkB and initiate X-ray analysis to set the stage for structural determination. The long-term goals of our structural biology efforts are to provide an atomic description of AlkB structure, and to uncover the mechanisms of selective modification of hydrocarbons. The structural information will help elucidating how the unactivated C-H bonds of saturated hydrocarbons are oxidized to initiate biodegradation and biotransformation processes. The knowledge gained will be fundamental to biotechnological applications to biofuel transformation of non-edible oil feedstock. Renewable biodiesel is a promising energy carry that can be used to reduce fossil fuel dependency. The proposed research capitalizes on prior BES-supported efforts on over-expression and purification of AlkB to explore the inner workings of a bioenergy-relevant membrane-bound enzyme**.**

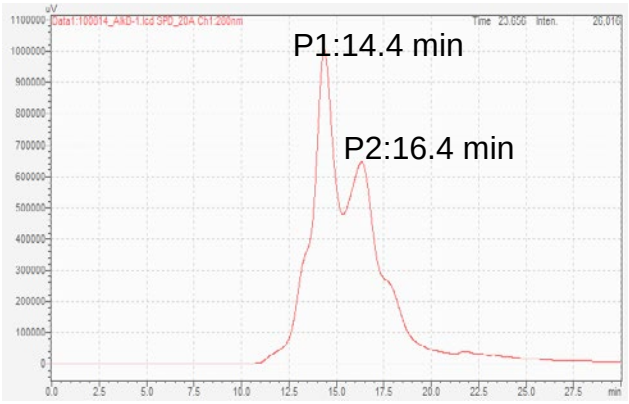
Science objectives for 2014-2016:

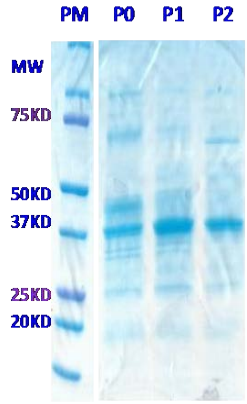
- Purify AlkB in crystallographic quantity and quality
- Identify initial crystallization conditions

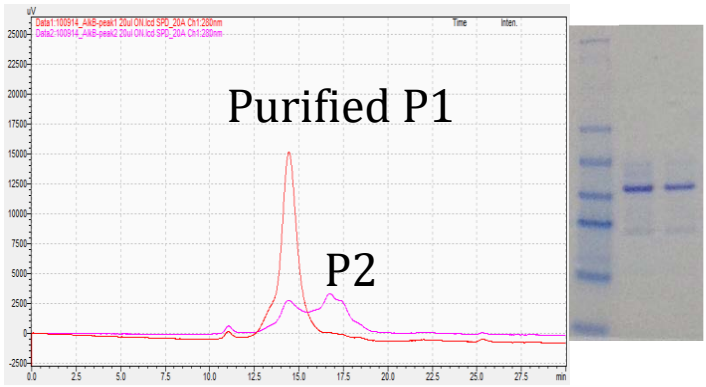
1) Recent progress and problems encountered

Purification We purified AlkB from heterologous over-expression in *E. coli* C43(DE)3 cells using auto-induction method. Membrane vesicles were prepared by multiple passages through a high-pressure cell disrupter (Microfluidizer) and collected by ultracentrifugation. AlkB in the membrane was solubilized by DDM and purified by Ni-NTA affinity chromatography using an AKTA Purifier system. AlkB eluted from the column was dialyzed to remove imidazole and His-tag was cleaved by overnight digestion using thrombin. The resultant full-length AlkB protein was subjected to size-exclusion HPLC analysis and SDA-PAGE analysis.

The sizing HPLC profile showed two major peaks at 14.4 and 16.4 min, respectively. SDS-PAGE showed that both peak fractions contained AlkB at around 37 kDa position with additional protein bands corresponding to contaminants and misfolded AlkB. P1 and P2 fractions were collected and re-run on sizing HPLC, showing a mostly mono-dispersed P1 profile (red trace). The accompanied SDS-PAGE analysis showed a purified P1 protein band with some minor contaminants. The P2 re-run on sizing HPLC (magenta trace) indicated that this fraction was not stable. Therefore, follow-up optimization was focused on conditions that promote the P1 peak.

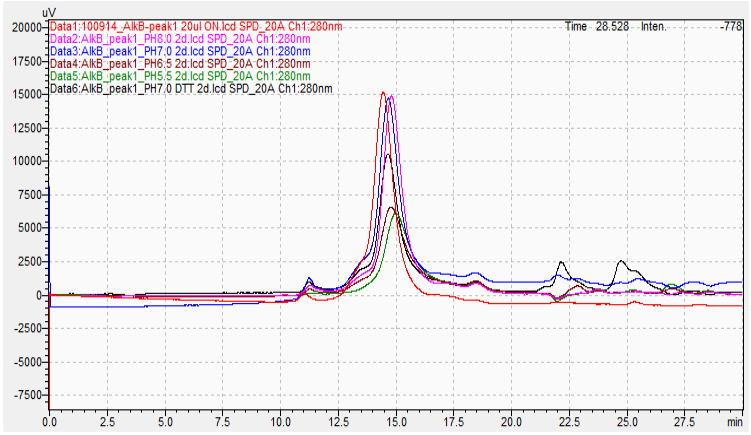






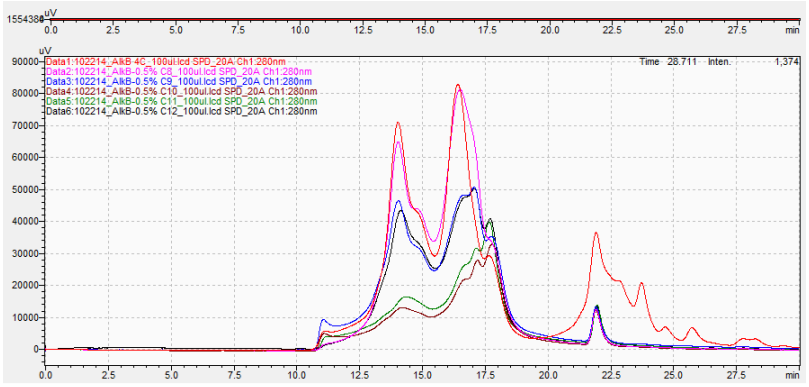
We first examined the pH effect on AlkB stability over a pH range from 5.5 to 8.0. The optimal pH was 7.0. At a lower pH of 5.5 or 6.5, AlkB was prone to precipitation. The stability at pH8.0 buffer appeared compatible with that of pH7.0.

Next, we examined the effect of substrate binding on AlkB. A series of alkane substrates including, C_8H_{18}, C_9H_{20}, $C_{10}H_{22}$, $C_{11}H_{24}$, $C_{12}H_{26}$ were incubated with purified AlkB at a concentration of 0.1-0.5% (v/v), and sizing HPLC analysis was performed using P1 peak profile as a stability readout. Compared with sample without any alkanes, adding 0.5% octane appeared more stable than other alkanes. However, none of these alkanes seemed to make AlkB sample significantly more stable.



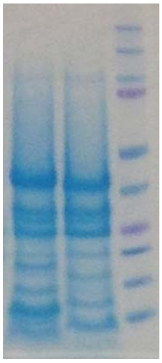
The lack of stabilizing effect of substrate binding prompted us to explore different

methods of substrate application to AlkB because we speculated that AlkB might bind alkane more effectively in membrane-bound state. Accordingly, we added alkanes of various chain lengths at different concentrations (0.1-0.5%, v/v) to the membrane pellet just before AlkB was solubilized so that alkanes could be partitioned into the membrane phase before detergent solubilization. However, adding alkane directly to the membrane made purified AlkB even less stable.



We also explored metallation of AlkB by adding ferrous iron to the culture media or purified AlkB, but no improvement of AlkB stability was found.

2) Analysis of encountered problems Although we could purify AlkB in a mono-dispersed form, the P1 fraction was only a minor species of total AlkB. As a result, the protein yield of AlkB in the P1 fraction was too low for crystallization screen. We believe that this problem is rooted in AlkB misfolding because the large majority of over-expressed AlkB was found in membrane pellets that were not soluble by DDM extraction. The appearance of the insoluble AlkB was similar to that of inclusion body of misfolded proteins. To overcome this problem, we tried many different conditions to optimize AlkB expression including using different media (Terrific Broth-TB, 2xYT media, media supplying with $FeSO_4$ and octane), expression temperature, IPTG concentration, and induction time and expression host cells. Our comparative analysis indicated that the best expression condition in our hands was the autoinduction method at 37 ^{O}C with C43DE3 host cells.



By some good luck, we accidentally uncovered the cause of multiple protein peaks in the purified protein sample. Previous studies showed that urea wash of the membrane vesicles helped removing contaminant proteins. We added a urea wash step before membrane solubilization, and to our surprise, we observed several additional small protein bands on SDS-PAGE. The molecular identities of these small protein bands were identified by N-terminus sequencing. They were all proteolytic products of AlkB, and three cleavage sites were mapped to the C-terminal domain of AlkB. This unexpected result indicated that the C-terminal domain of AlkB became partially unfolded upon urea exposure. This unfolding event made AlkB susceptible to protease digestion, resulting in the P2 peak on sizing HPLC. Accordingly, we added protease inhibitors throughout the purification process. The protease inhibitor effectively protected AlkB and significantly increased the P1 peak, corresponding to the intact AlkB protein.

With protease inhibitor and several other modifications, we could obtain sufficient

amount of AlkB in P1 fraction for crystallization screen. However, our progress was slowed down again by another technical difficulty. The purified AlkB was prone to forming protein aggregates when the sample was concentrated. Since high protein concentration is a prerequisite for effective crystallization screen, we must identify additional stabilizing conditions to prevent AlkB from aggregation at high protein concentrations.

A working hypothesis for AlkB aggregation was the partial misfolding of the C-terminal domain, which is highly susceptible to proteolytic cleavage. One possible cause of misfolding is the over-delipidation of AlkB when it was extracted from membrane by DDM. Although we have already taken extra precaution to prevent over-delipidation, extensive detergent wash seems inevitable because the association of contaminant proteins with AlkB is very strong. To avoid detergent wash all together, we will use a novel detergent-free purification method developed by John Shanklin. Shanklin lab will provide the protocol and necessary training for us to try this novel purification method.

In the last year of the DOE support, we tried two different ways to avoid over-delipidation of AlkB during the purification process. First, we minimally washed the protein on the affinity column and reconstituted the partially purified AlkB into proteoliposomes. After washing the proteoliposomes free of soluble contaminant proteins, we re-solubilized AlkB using minimal amount of detergent. While this method improved the protein stability and purity, the protein yield was low because of the extra reconstitution step. Second, we tried Shanklin's detergent-free purification method, but met with limited success.

3) Publications that acknowledge the support of your DOE BES grant during the past year.

None