Lecture #4

2/11/04 Lecture 4

Recent FAS references (not required reading)

Science (2000) 288, 2379
PNAS (2003) 100, 12624
PNAS (2002) 99, 138 (cryoEM)
J Lipid Res (2003) 44, 1 (model ACP dock)

3) Structure (continued from lecture 3)
X-ray structures of individual FAS domains have been solved, but there is no structure of the entire protein. Determining the structure is probably difficult because of the flexibility of the protein. There is a structural model by cryoEM methods at 19 angstroms resolution.

Domain- section of protein that has a single catalytic activity

Experimental methods for elucidating FAS structure:

I. Proteolysis

Limited proteolysis divides the FAS protein into 3 parts (I, II, III)

1. I. KS, AT, and DH domains
2. II. dimer interface, ER, and KR
3. III. TE

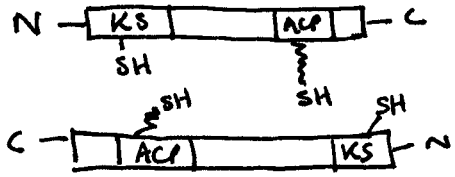
Proteolysis allows one to simplify the analysis. Researchers have used creative ways to assay the activity of I, II and III and hence started to define the domain structure.

With current technology, probably the first step would be to look at the gene sequence and search for sequence homology. Using this approache and the methods used in T & D you could tell from the E. coli KS gene sequence that the N-terminal domain of FAS is in fact a KS.

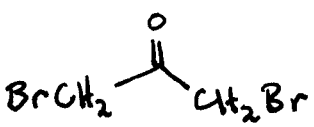
II. Chemical Crosslinking

How are the domains organized? What is the tertiary and quarternary structure? Crosslinking with dibromoacetone provided the model of head to tail interactions of the mammalian FAS homodimer seen in the text books.

H to T interaction



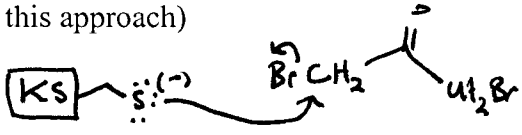
Non-specific alkylating agent

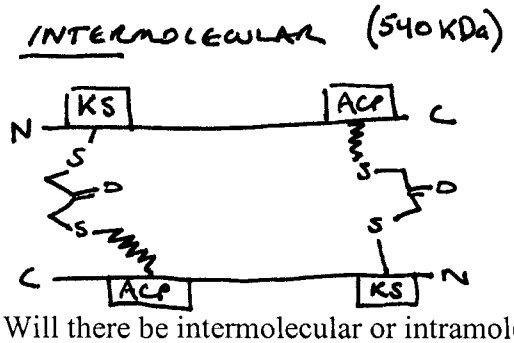


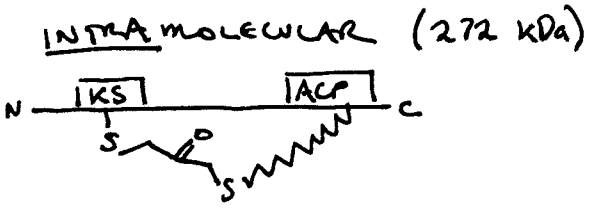
Is the cys in the KS domain close in three dimensional space to the thiol of the ACP on the same protein, or is it closer to the thiol of the A2 in the other monomer?

Why will the cysteines act as nucleophiles? Normal pKa of cys is 8.5-9
pKa in the active site is often perturbed- pKa can be as low as 4! Cysteine thiolate is the reactive species. The use of bromoacetone, a non-specific alkylating agent requires that your thiolates are more nucleophilic than imidazole from histidine. (Of course, one never has absolutely specific labeling using this approach)

LEARN THE pKa's of the AMINO ACIDS!





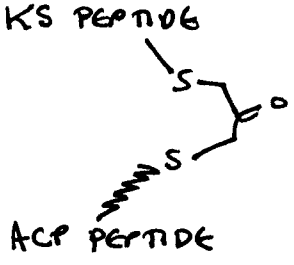


Will there be intermolecular or intramolecular crosslinking?

You can distinguish between these two results by running an SDS PAGE gel. This method separates proteins based on the difference in their molecular weights. The monomer is 270 KDa and the dimer is 540 KDa.

You can also vary the length of the crosslinking agent, dibromoacetone has a short distance between the two CH_2Brs. If the thiolates were farther apart, then crosslinking would not be observed. Often one uses crosslinkers with variable spacing between the reactive positions.

How do you determine to which amino acids, in which peptide contexts, the crosslink is attached? Which thiols are modified?
-Use radioactive labeling of the crosslinking agent (usually ^{14}C or ^{3}H, both beta emitters)
-then digest the crosslinked protein with a protease, separate the peptides by HPLC, find the labeled peptides by scintillation counting and sequence the radiolabeled peptides. Also quantitation of label recovery tells use how efficient alkylation of cysteinse and thilate of pantetheine actually is.



When this experiment was first performed, they found only the **inter**molecular product- leading to the acceptance of the head to tail model

When the experiment was repeated recently, the **inter**molecular product was still predominant, but they did find some **intra**molecular crosslinking as well

How flexible and floppy is the protein? How much is it moving around?

III. Map domain interactions

VS Rangan, AK Joshi, S Smith. *Mapping the Functional Topology of the Animal Fatty Acid Synthase...* Biochemistry **40(36)** 2001, 10792-10799. See the cartoon that gives the subunit orientation from the Smith SDM and heterodimer experiments.

This reference is a good example of actual experimental data and the often observed complexity. Learning to critically evaluate primary data is an important skill. Think about the primary data and experiments when you look at the "pretty cartoons" in textbooks- what evidence are these models based on?

In this experiment they determined that the ACP domain interacts with the KS and MAT domains of either monomer. However, the ACP only interacts with the DH, ER, KR, and TE on the same monomer.

Two techniques are described in this paper:
1) protein tag - genetically coded
-allows you to purify protein easily and "painlessly"
-separate heterodimers and homodimers. This method requires that the two sets of homodimers can be reorganized to form a 1:2:1 mixture of homo-hetero-homo dimers.

Where do you put the tag? Usually it is put at the C or N terminus of your proteins- these positions are usually on the surface of the protein and close to each other

1. Histidine tags (6- 10 histidines)- This tail binds to a Ni affinity column, His coordinates to metal which is coordinated to the column. This is an equilibrium process. The protein is often eluted with imidazole, but some times high salt or EDTA (Ni chelator) are used.

2. FLAG-tag, HA-tag, myc-tag All peptide sequences which are recognized by monoclonal antibodies (Mab) that are commercially available. Unfortunately, removal of your protein from the Mab often requires strong acid and the protein is denatured.

These genetically encoded tags have evolutionized protein purification

2) Site-directed mutagenesis (SDM)
Replace any naturally occurring amino acid with another natural amino acid. The methods to make mutants will be discussed in T &D.

SDM is used to specifically inactivate the catalytic activity of a defined domain. This inactivation can be done because we understand a lot about the chemistry of each reaction.

You generated a tagged-homodimer with one domain inactivated and a second and differently tagged-homodimer with wt or a differently inactivated domain.

You generate and purify a heterodimer from the two homodimers and then study the properties of your newly generated heterodimer.

Example, in the KS cys - > ala (the thiol in the active site, essential for covalent catalysis is replaced by a methyl group). This mutation destroys the KS domain activity.

Second example: Change in the TE domain a ser to ala , kill activity. The serine is essential for covalent catalysis.

It is easy to inactivate domains that involve covalent catalysis. Other domains such as ER, KR are harder to inactivate. Both ER and KR are NADPH binders and we know enough about the structure that binds to NADPH to be able to mutate residues within the binding site to greatly reduce activity

If you start out with all homodimers, they would equilibrate over time (1 week, 4 deg C) XX XX YY YY -→ XX XY XY YY Note, cold makes the hydrophobic interface of the homodimers weaker.

End with a 1:2:1 ratio of homo:hetero:homo

In this experiment they were able to separate out heterodimers based on the protein tags

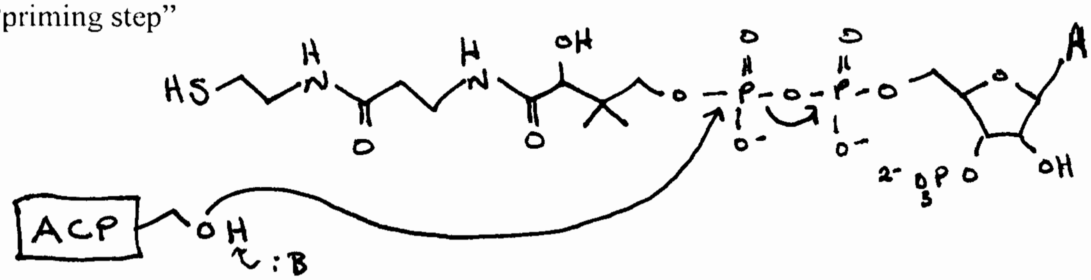
They concluded that the ACP domain interacts with the KS and MA domains of either monomer. However, the ACP only interacts with the DH, ER, KR, and TE on its own monomer.

4) Chemistry
We will now discuss the chemistry of FAS as a foundation for understanding PKS and NRPS

Swinging pantathiene arm - need a specific enzyme to put on this arm

1. Post-translational modification

"priming step"



ACP domain (a small protein) is inactive without the swinging arm
"ACP synthase" or more generally called, a "phosphopantethenieyl transferase"
PfTase, modifies the serine in the ACP with CoA