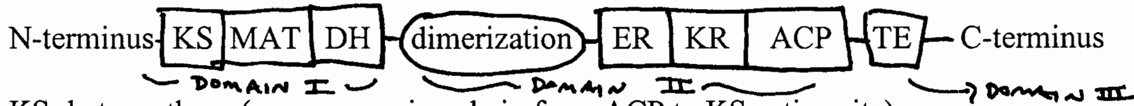
TD2
Reference: S.Smith, Biochemistry (2001) **40**, 10792

I. Background

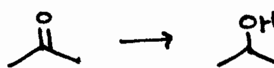
mFAS = mammalian FAS (homodimer- made up of two 272kDa monomers)
each monomer has the following composition: 3 domains, 7 modules



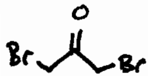
KS=ketosynthase (moves growing chain from ACP to KS active site)
MAT= malonyl/acety; transferase (loads ACP with malonyl or acetyl extenders)
DH= dehydrase

ER= enoyl reductase

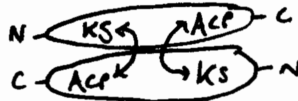
KR= ketoreductase



ACP=acyl carrier protein (has swinging pantathiene arm)
TE= thioesterase (releases product as free acid)



Crosslinking experiments have shown head to tail dimerization of monomers (early experiments showed only intermolecular crosslinking)



II. Question

What is the spatial arrangement of the modules & domains in the mFAS homodimer?

Can a growing chain on the acp pf one monomer use the KS, MAT, DH, ER, KR, and TE domains on the same monomer only (intramolecular) or can it use the other monomer (intermolecular)?

III. Approach

"Mutant Complementation"
Use site directed mutagenesis to knock out activity of a domain on one monomer
Then, assay activity of a homodimer

Ex: KS^- = mutant with no KS activity, KS knockout
Knockout ACP on monomer A, Knockout KS on monomer B

If there is activity in the ACP^-, KS^- homodimer, then the ACP of B must interact with the KS of A
In other words, ACP^- "complements" KS^-

Want to make a complete data set:
ACP^- complements KS? MAT? DH? ER? KR? ACP? (of the other monomer) TE?

IV. Technique 1: Site directed mutagenesis

1. Look at active site, select a single amino acid to mutate to kill enzyme activity

ex: Cys-> Ala
Ser-> Ala
Gly-> Tyr (disrupts NADH binding loop of ER)

2. obtain gene (DNA) for enzyme of interest (usually on a plasmid)
3. design "oligos" (synthetic DNA) that contain the mutation & anneal (imperfectly) to the target site
4. PCR amplify the DNA; digest with a special enzyme that cleaves only template (original) DNA; introduce into bacteria
5. re-isolate DNA from bacteria, check for mutation by sequencing

Modification at DNA level to change the protein product

V. Technique 2: Making heterodimers

A. Affinity purification tags

1. Affinity purification tags are introduced at the DNA level (ex. FLAG tag)
2. Express protein with peptide tag
3. Run through a column w/ beads that bind the tag (ex. Anti-FLAG antibody) Protein will stick to the column
4. wash column, to elute everything w/out tag
5. elute protein w/ special buffer- competing ligand or change pH etc

Gives purifed protein with tag!

Another example of a tag is a His6 tag (6 his residues in a row)
This tag binds to a different type of column - Ni-NTA
(Nickel-nitrylotriacetic acid) that has two open coordination sites to bind hisitidines
to elute his-tagged protein, wash with imidazole (competing ligand) or EDTA (metal chelator)

B Use tags to prepare heterodimers

1. prepare X-FLAG purified protein
2. prepare Y-HIS purified protein
3. mix together in 1:1 ratio in conditions where they will equilibrate, to give

X-FLAG X-FLAG (homodimer) X-FLAG Y-HIS(heterodimer) Y-HIS Y-HIS(homo)

4. run FLAG column, discard flowthrough (Y-HIS Y-HIS)

elute FLAG dimers (X-FLAG X-FLAG, X-FLAG Y-HIS)

5. run Ni-NTA column, discard flowthrough (X-FLAG X-FLAG)

elute pure heterodimer! X-FLAG Y-HIS

VI. Results

ACP$^-$ complements KS$^-$ & MAT$^-$
does not complement DH$^-$, ER$^-$, KR$^-$, &ACP$^-$
weakly complements ER$^-$

Therfore, ACP on one monomer can use KS and MAT modules of the other monomer
Cannot use DH, KR of other monomer
Can barely use ER of other monomer

Definitions and procedures:

Plasmids- circular doublestranded DNA

Used for manipulation of DNA
-amplification
-cloning
-mutagenesis
-sequencing

Plasmid= self-replicating circular extrachromosomal DNA found in *E.coli*: Usually encodes for antibiotic resistance or production of restriction enzymes

Re-engineered versions for molecular biology have 3 common features:
1) cloning site, many restriction sites all in one location for pasting in genes of interest
2) replicator (ori) site, site at which DNA replication begins, confers self-replicating quality
3) resistance gene/ selectable marker
ex. Gene for B-lactamase, which cleaves antibiotic ampicillin and therefore confers antibiotic resistance to host bacteria

<u>To introduce plasmids in *E.coli*</u>
"transformation"
-apply electric, chemical or heat shock to bacteria in presence of the plasmid
-plasmid goes into some bacteria
-plate onto agar + antibiotic (ex. Ampicillin)
-only bacteria containg the amp. resistance gene (and therefore the plasmid) will survive, all untransformed bacteria die
<u>To re-isolate plasmid DNA from E.coli ("mini-prep", "plasmid prep")</u>
-grow up single colony of E.coli in antibiotic containg media
-harvest bacteria from culture
-use base to lyse the bacteria
-add acid to meutralize-> protein precipitates out of solution
-centrifuge to remove precipitant-> load crude DNA only column (Resin= anion exchange resin)
-rinse column with ethanol to remove small molecules
-elute DNA with water

PCR: Polymerase chain reaction
Purpose: to make a huge number of copies of a gene (or any piece of ds DNA)

A PCR reaction contains

1. DNA template-what you want to make copies of
2. Forward oligo/primer- short singlestranded DNA (15-40nt) that anneals to the beginning (5' end) of the segment of template you want to amplify
3. Reverse oligo/primer – same as forward, but anneals to 3' end of template
4. dNTPs (for making new DNA)
5. DNA polymernase (Taq, Pfu, DeepVent, etc) enzyme that catalyzes DNA synthesis (5'->3')
6. Mg^{2+} containing buffer- for polymerase activity

A PCR machine cycles to PCR reaction through different temperatures

95 deg- denaturation of doublestranded (ds) DNA template into singlestranded (ss) DNA
42-62 deg – annealing of primers to ssDNA template
72 deg- synthesis of new DNA by polymerase enzyme
Repeat cycle 20-30 times

Exponential DNA synthesis
1 DNA molecule-> $2^{30} = 10^9$ = 1 billion DNA molecules in 30 cycles

See page 114 of Voet&Voet Biochemistry for pictures

Site-directed mutagenesis

1. isolate plasmid with gene of interest – plasmid has methylated DNA (from bacteria – methyl transferases use SAM to methylate cysteines)
2. Anneal to mutagenic oligos (complementary to original gene sequence except at site of mutation)
3. PCR with Pfu polymerase
4. Use DpnI restriction enzyme to digest original methylated DNA
(PCR product is unmethylated)
5. transform into bacteria
6. isolate DNA and sequence to be sure the desired mutation is incorporated