TD 12
Characterization of DnaJ substrate specificity
Reference: EMBO J., 2001, 20, 1042-50, Bukau et al.

Techniques: peptide scanning; western/immunoblot

I. Background

Hsp 70 Chaperones: participate in protein folding, refolding of misfolded proteins, protein translocation, complex assembly/disassembly. Can act either alone or in concert w/ a co-chaperone.
Hsp 40 Co-chaperones: assist Hsp 70 chaperones

DnaK= *E.coli* Hsp70 type chaperone

DnaJ=*E.coli* Hsp 40 type co-chaperone

*Note- DnaK and DnaJ are both PROTEINS.

Question: What is the role of DnaJ?
Observations:
-DnaJ itself can bind unfolded proteins
-This binding can prevent protein aggregation-> in this sense, DnaJ is a chaperone on its own.
-But, DnaJ requires DnaK to refold misfolded proteins
-Domain structure (DnaJ) : 376 amino acids
From N-terminus
J domain – amino acids 1-78, interacts with DnaK
G/E domain- contributes to stimulation of ATP hydrolysis (of DnaK)
Zn binding site, amino acid ~144
C-terminal domain- amino acids 144-376, binds protein substrates

Model: DnaJ first binds to unfolded protein substrates, and then hands them over to DnaK, triggering ATP hydrolysis as it delivers substrate to binding cavity of DnaK
If this is true, what is the substrate specificity of DnaJ and how does it compare to that of DnaK?

II. Overall approach

Hartl method (GroEL): Immunoprecipitation (IP) to screen entire genome
-technically difficult
-identifies protein substrates

Here (DnaJ): peptide scanning of known protein substrates
-technically more straightforward
-not *in vivo*- less relevant
-identifies specific regions of interaction within known protein substrates

III. Technique 1: peptide scanning

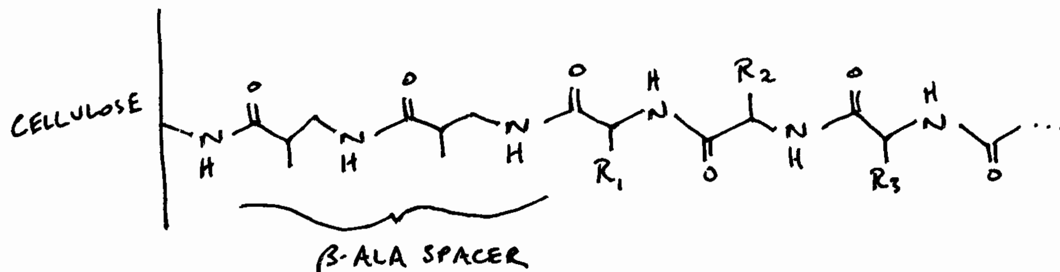
Known substrates of DnaJ: DnaA, λP, p53, luciferase, RepE etc (14 proteins total)

To determine which regions of DnaA interact with DnaJ, make a bunch of DnaA derived peptides

Peptide 1= amino acids 1-13
Peptide 2= 4-16
Peptide 3= 7-19
Etc…each peptide is 13 amino acids long
Each peptide overlaps with previous peptide by 10 amino acids

Synthesize on cellulose membrane (Remember: chemical peptide synthesis C->N vs. ribosomal N->C)
Small scale, solid support, array delivery = faster, less expensive

Each peptide is attached to the cellulose membrane by a linker composed of two beta-alanine linkers



For DnaA (~450 amino acids) make a matrix of 450/3= 150 peptides immobilized on a cellulose sheet.
Peptide immobilized on cellulose -> add DnaJ; incubate -> rinse off unbound -> use western blot to quantify amount of DnaJ that has stuck to peptide

Controls:

1) Negative control = cellulose + beta-Ala linker only
2) Positive control = peptide known to bind tightly to DnaJ

Big assumption of this approach: DnaJ binds to certain amino acid sequences regardless of context. What about environment? Secondary structure?

IV. Technique 2: Western blot/ immunoblot

*Highly sensitive (and sometimes quantitative) method to detect specific proteins using antibodies

Requires: antibody specific to protein target (DnaJ)
Reporter to detect bound antibody (often enzymatic)

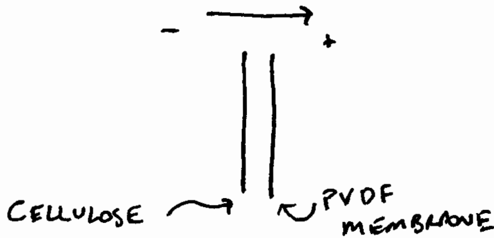
Procedure:

↓ Cellulose-peptide + DnaJ

↓ Some DnaJ still unbound, some cellulose-peptide-DnaJ

↓ Rinse off unbound DnaJ

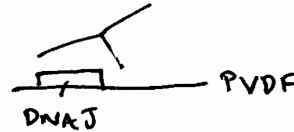
Transfer DnaJ to PVDF membrane (general protein sticky surface)
Transfer electrophoretically



DnaJ transfers onto PVDF membrane. Once there, it is stuck for good.

↓ Block PVDF membrane with serum or milk (Passivate the surface of the membrane, so it is no longer sticky)

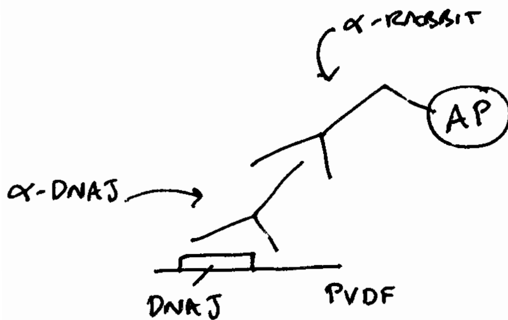
↓ Add anti-DnaJ antibody, it binds to DnaJ

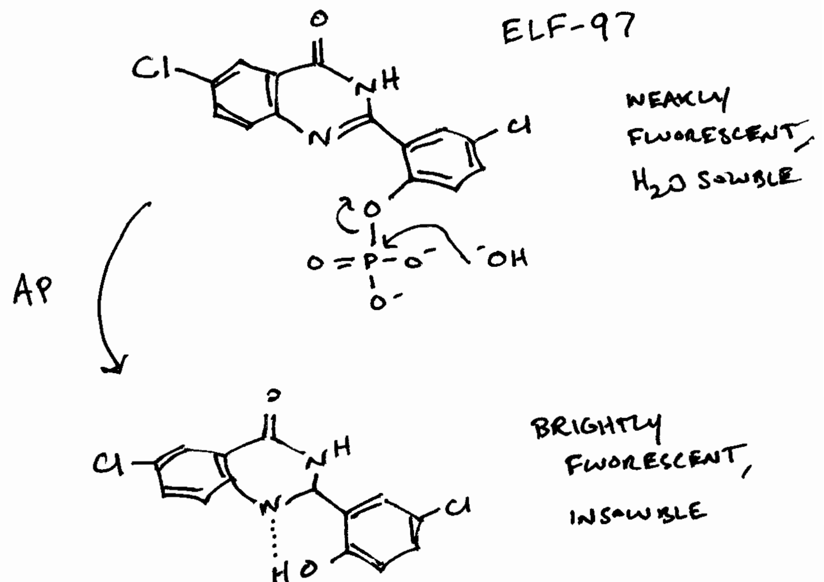


↓ Rinse off unbound antibody

↓ Add anti-rabbit secondary antibody conjugated to alkaline phosphatase (AP) reporter anti-rabbit secondary antibody binds to constant region of anti-DnaJ antibody (from rabbit)

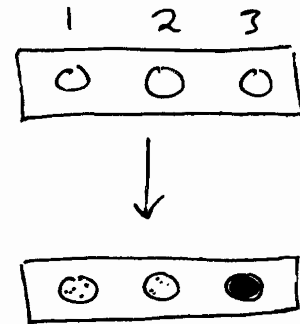
Add fluorogenic substrate of AP, ELF-97
ELF-97 is weakly fluorescent and water soluble, it reacts with AP to give brightly fluorescent and insoluble product that deposits at site of DnaJ-antibody-antibody





Controls:

1) Blank cellulose
2) Beta-Ala spacer only
3) Beta-Ala spacer linked to high binding DnaJ peptide



Perform procedure on each control exactly as described above.
Expected results:
1) background fluorescence
2) background fluorescence
3) intensely fluorescent spot (indicates DnaJ binding)

V. Peptide scanning results

See **figure 1**. from EMBO J., 2001, 20, 1042-50, Bukau et al.

Each position = 13 mer peptide from substrate protein sequence
Dark spot= DnaJ binds to that peptide

Observations:
-a range of binding affinities observed (lightness or darkness of spot)
-Some binding regions are less than 13 amino acids, some are greater (number of peptides in a row that bind DnaJ)
-some proteins have more interaction sites than others (p53> λP)

VI. Analysis of peptide targets

See **figure 2** from EMBO J., 2001, 20, 1042-50, Bukau et al.
See 2A
-looked at 1633 peptides whose amino acid composition matches that of entire genome

see **2B**
-white bar = DnaK binding peptides from a previous study
-black bar= DnaJ binding peptide
-bars that go above the mid-line indicate the DnaJ binding peptides were enriched in that amino acid, bars below indicate that amino acid is depleted
-DnaJ binders are enriched in aromatics (F,W,Y) and hydrophobics (I,L,V)

See 2C

To make this graph, 62 DnaJ binding peptides are aligned, with the most N-terminal hydrophobic/aromatic residue anchored at position 10 (This explains why the x-axis contains 22 positions, as well as the large hydrophobic/aromatic bar at postion 10)
black bars= hydrophobic/aromatic residues
white= acidic
grey = basic

Notice that hydrophobics/aromatics are enriched from position 10 to 10+7
This alignment allows them to conclude that DnaJ recognizes a hydrophobic stretch of ~8 amino acids
(in comparison, from another study, we know that DnaK recognizes a ~4-5 amino acid stretch)

VII. Overlap between DnaJ and DnaK substrates

See Figure 3 from EMBO J., 2001, 20, 1042-50, Bukau et al.
3A.
Luciferase-derived peptides
Black= DnaK binding affinity
White = DnaJ binding affinity
->some differences are apparent
3B.
Peptides that bind DnaJ but not K contain several aromatic/hydrophobic amino acids with acid amino acids in between
3C.
Competition assay;
DnaK peptide complex at time D
->add DnaJ and watch fluorescence decrease

Conclusion:
6/7 of DnaK peptides also bind DnaJ
¾ of DnaJ peptides also bind DnaK (J is more promiscuous)
DnaK specific peptides have shorter hydrophobic stretch
DnaJ specific peptides have extended hydrophobic stretch with some acidic amino acids (D,E) in between
DnaK+ DnaJ can compete with each other for peptide binding

VIII. Importance of amino acid chirality

See figure 4
DnaJ doesn't care if peptide is D or L stereochemistry,
DnaK only accepts L (natural) peptides, not D
-Is the mechanism of binding different?
Maybe DnaJ relies mainly on sidechain contacts, and the backbone orientation is not as important

IX. Model for DnaJ function

See figure 5
A.Shows cartoon of general properties of DnaJ/DnaK substrates
C. Shows two models for interaction
1) Handover – 1 binding site, DnaJ and DnaK bind to same site on substrate
2) Recruitment – 2 binding sites, DnaJ and DnaK may both be bound at once to same substrate

Data from this experiment is not sufficient to exclude either model