7.88 Lecture Notes - 14
7.24/7.88J/5.48J
The Protein Folding and Human Disease

Chaperonins

- **Chaperonins and Protein Misfolding**
- **Segue into GroEL**

Misfolding >> Kinetically trapped aggregates

A. Chaperonins

Just three experiments:

1) original RUBISCO set a, b, c
2) two stage Rubisco
3) Perhaps 1 generalized mitochondrial import experiment

Chaperonins: Protein families that help proteins fold within cells:

- Maintain unfolded states for protein export or import
- Assist chain folding correctly within (stressed) cells
- Inhibit formation of misfolded, aggregated states
- Refold as proofreading function
- Select/unfold them for degradation

The chaperonins were originally identified through convergence of three tracks:

- Plants: Rubisco synthesis in chloroplasts; Newly synthesized large subunit associated with Large Subunit Binding Protein:
- Phages: Host genes required for phage morphogenesis.
- Eukaryotes: Heat Shock genes turned on in fruit flies in response to heat

Ubiquitous: present in all living cells that have been examined; highly homologous.

Many other Stress Genes

- O2,
- toxins such as alcohol,
- radiation

Classes of **Stress Proteins**

HSP 60s	E. coli	Yeast	Mammals
HSP 70s			
HSP 90s			
HSP104			

Chaperone proteins and their known functions in the cell

Subcellular Location	Organism	Component	Subunit Mr (kDa)
The hsp 70 family			
Cytosol	*E. coli*	DnaK	69
	Yeast	Ssa1-4p	69-72
	Mammals	hsc70	
Mitochondria	Yeast	Ssc1p	70
Endoplasmic reticulum	Yeast	Kar2p	78
		Bip, Grp78	70
The hsp60 family			
Cytosol	*E.coli*	GroEL	58
		GroES	10
Mitochondrial matrix	Fungi, mammals	hsp60, hsp58	58-64
		hsp10	10
Chloroplasts	Plants	Rubisco subunit binding protein	61 (α-chain) 60 (β-chain)
SecB	*E. coli*		17
The hsp90 family			
Cytosol	Mammals	hsp90	90

HSP60 class of chaperonins:

- GroEL/S
- Cpn60/cpn10
- HSP 60

Purified by Roger Hendrix J. Mol. Biol (1989) **129** 359 - 373.

GroEL/ES
22 S
14 mer of subunits 60k GroE Large
+ 7mer of 14k Gro E Small.

Tetradecamers

- Double barrel, cyclic seven-fold symmetry, and two fold reflection symmetry:
- 7 fold cyclic cap sits on one end in physiological mode

Remind you that we have already shown that

- Folding Intermediates > Aggregate
- Aggregation is generally kinetic trap for the chains
- Folding intermediates likely to be intrinsically thermolabile with respect to native

1) Experiment 1:

Long history of difficulty in understanding biosynthesis and in refolding protein in vitro from Chloroplasts.

Best defined experimental work is function of E. coli GroE, on hetrologous substrate,

RUBISCO - **Ribulose bisphosphate decarboxylase.**

In higher plants this **L8/S8** structure.

However in cyanaobacteria (Anacystis nidulans) large subunit dimer is a catalytically active form.

Rubisco from Rhodospirillum rubrum - Cyanobacterial RUBISCO refolds very poorly by itself in physiological temperature range. In fact can be refolded by itself at low temperature; yield very sensitive to temperature:

Dupont group: co-express with plasmid overexpressing GroE proteins, get increased formation of active enzyme in vivo.

The mechanism of this rescue is described in
"Reconstitution of Active Dimeric RUBISCO from an unfolded state depends on two Chaperonin Proteins and Mg-ATP. Pierre Goloubinoff, John T. Christeller, Anthony Gatenby and George Lorimer Nature, **342**, 884-889 (1989).

Denatured proteins easily distinguished from native by circular dichroism

Urea and guanidine denatured states similar; low Ph state quite different; perhaps molten globule-like, but not yet determined.

Depends on ratio of unfolded to GroEL: too low, no dimers, too high, aggregation:

Viitanen, P.V., Thomas H. Lubben, Janet Reed, Pierre Goloubinoff, Daniel P. O'Keefe and Geroge H. Lorimer Chaperonin-Facilitated Refolding of Ribulosebisphosphate Carboxylase and ATP hydrolysis by chaperonin 60 (groEL) are K+ dependent
Biochemistry (1990) 29, 5665-5671.

Refolding yield vs. temperature absent GroEL

If refold at 10°C, slow but high yield.
Refold at 25°C: Less than 1% recovered! 99% aggregated

Their efforts to recover enzymatic activity after dilution out of denaturant were unsuccessful.

However, if they diluted into solution containing both Gro proteins in high concentrations plus ATP, then recovered activity.

Refolding at 15°C with and without GroEL:
Rates similar, but different yields.

This suggest that chains proceeed to common intermediate prior to chaperonin rate-determing step.

Now if examine yield versus ratio, for example varying R-U concentration, they found a distinct maximum.

On low side have problem of concentration insufficient for dimerisation;

On high side chains end up aggregated, and these species not rescued by reaction conditions.

2) Exp 2

Clearest evidence that chaperonin is recognizing kinetic intermediate comes from two step dilution experiment, where dilute

incubate varying times

Then incubate with chaperonin solution.

If dilute directly in to GroL, recovery is close to 200nM. However Recoverability decays quickly with time:

So Ri >>>>Aggregate;

However, does not continue; because critical aggregation concentration
Nucleation growth >> growth

Recoverability falls off rapidly with time after initial dilution.

So target species is **transient** species.

Overall mechanism;
Intermediates recognized, not native state, not fully denatured

Mechanism to be reported in detail in student papers: Overall mechanism; Flow chart
: binding into lumen:

Binding of GreS forming a Cap

Unfolding within lumen

Further folding in lumen;

Uncapping:

Release from lumen

Binding to other ring

Capping etc

HSP60 class of heat shock chaperonins is preventing off pathway aggregation of folding intermediates:

HSP 70 DNAK
N-terminal 45kdATPase domain
18kD peptide binding region
peptide bound in extended form

In prokaryotes found as complex with DNAJ and GrpE, regulate activity

Problems associated with proline isomerization

3) Problems associated with prolyl isomerization

During protein synthesis, all peptide bonds are synthesized in the trans configuration:

However, upon denaturation in urea of GdnHCl, prolyl peptide bonds can isomerize to cis. This has a half time of about 20 minutes at room temperature.

In order to refold the chains with cis prolyl bonds have to re-isomerize to the trans state, before they can refold;

This can rise to heterogeneity generating
fast folding species
slow folding species;

If multiple bonds, then multiple species, with different lag times:

Experimentally double jump experiment; examine dependence of refolding on time of incubation in denaturant: If find dependence, evidence that this is a problem;

With cells, a class of enzymes prolyl isomerases: >> presumably deal with this problem where it comes out: but how recognize species, how function, complex:

Research Report Topic: presence of ATP and Mg, generate properly folded actin

4) Mitochondrial Import CHAPERONINS

Mitochondria have two membrane systems and contain the proteins involved in oxidative phosphorylation.

Though mitochondria have their own DNA and code for their own

- Most of proteins within mitochondria come from outside the organelle;
- Synthesized on cytoplasmic ribosomes; generally with a targeting presequence
- travel to mitochondrial surface;
- Transported inside
- mitochondrian presequence clipped off by a metal dependent processing enzyme in the matrix and
- protein folds back up again

A number of proteins on outer membrane are involved in recognition;

To pass through import channel protein must be unfolded;

Therefore

- if traveled through cytoplasm in folded state, has to be unfolded under fully native conditions;
- Or travels in unfolded state;

Cytoplasmic chaparonins and other proteins may maintain and carry protein in partially folded state:

Most populated in cytosol are 70k class.

5) Folding and Import Apparatus within mitochondrion

M have HSP 60; ts mutants in hsp 60 defective in assembly of a number of m proteins that are found in supramolecular complexes; F1 ATPase

Ostermann, Joachim, Arthur L. Horwich, Walter Neupert, and F.-Ulrich Hartl. "Protein Folding in mitochondira requires complex formation with hsp60 and ATP hydrolysis." *Nature* 341 (1989): 125-130.

Use as reporter Dihydrofolate reductase; cytosolic enzyme, - in cytoplasm folds to give protease resistant activity;

When fused to presequence, can cross membrane and fold inside mitochondrian to give protease resistant activity.
Fusion 1-69 of subunit 9 of F0ATPase -3aa linker - N term of complete DHFR.

This group had previously shown that
rate limiting step in transport >>unfolding of DHFR across M membrane. 66 residues of presequence are clipped within in two step process.

6) Is refolding within mitochondria energy dependent?

Experimental approach somewhat complex:

- Synthesize hybrid in reb blood cell lsyis, so radiolabeled.
- Precipitate by NH4SO4,
- dissolve in 8M urea, so denatured.

Experiment:

- Incubate with intact mitochondira;> rapid import, if M are energized
- Follow kinetics of refolding inside mitochondirion;
- Quench reaction by uncouplers of ATP synthesis and cooling
- Assay for inside by permeabilizing the mitochondiria with detergent digitonin ;
- Add protease ;

- protein stuck outside is unclipped and protease sensitive; Now assay protease resistant mature DHFR

Import Process extraordinarily efficient; Within 45 seconds at 25oC more than 90% of chains are translocated into mitochondria; However only 30% has reached protease resistance stably folded state at this time. By 3 minutes, 70% is stably folded.

Things slowed down at 10°C

Now these mitochondria making their ATP from NADH, which is channeled directly into respiratory chain.

Deplete Ms of ATP by adding apyrase, which hydrolyzes ATP - or non hydrolyzable ATP analogues.

Result: Import proceeds with high efficiency but construct doesn't get protease resistant.

7) Is folding reaction Hsp60 dependent?

Using same general protocol, after 5 minute incubation:

- Prepare extract of matrix,
- run through sephacryol 300 column sizing column.
- much of DHFR is in mature conformation, slow, protease resistant
- About 10% behaves as 700k complex. This materisal is protease sensitive.
- If deplete of ATP, this peak of the order of 60% of DHFR chains if Mitos had been depleted of ATP.

High molecular weight peak contains HSp 60.

Perform similar experiment with natural precursor; iron sulfur protein component of ATPase; cofractionates with Hsp60 from digitonin extract of matrix.

8) Are Hsp60 bound molecules foldng precursors?

If this is physiological complex, access to ATP should result in release of precursors from complex and appearance of native stably folded molecules;

Import fusion into ATP depleted Ms

- Prepare matrix extract
- Add ATP
- Now assay by Sephacryl;

- Distribution of Hsp60 relatively unchanged
- Mature length DHFR moves from fast sedimenting to its own position and becomes protease resistant.

Kang, Pil-Jung, Joachim Osterman, Jeffery Shilling, Walter Neupert, Elizabeth A. Craig, and Nikolaus Pfanner. “Requirement for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins.” *Nature* 348 (1990): 137-143.

In yeast, 8 hsp 70's. Saccharomyces cervisiae. One, ssc1p is located in mitochondrian. matrix.

They isolated a temperature sensitive mutant of yeast M hsp70.

At high temperature cells accumulated precursors of hsp60, hsp70, and FATPase subunit.

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