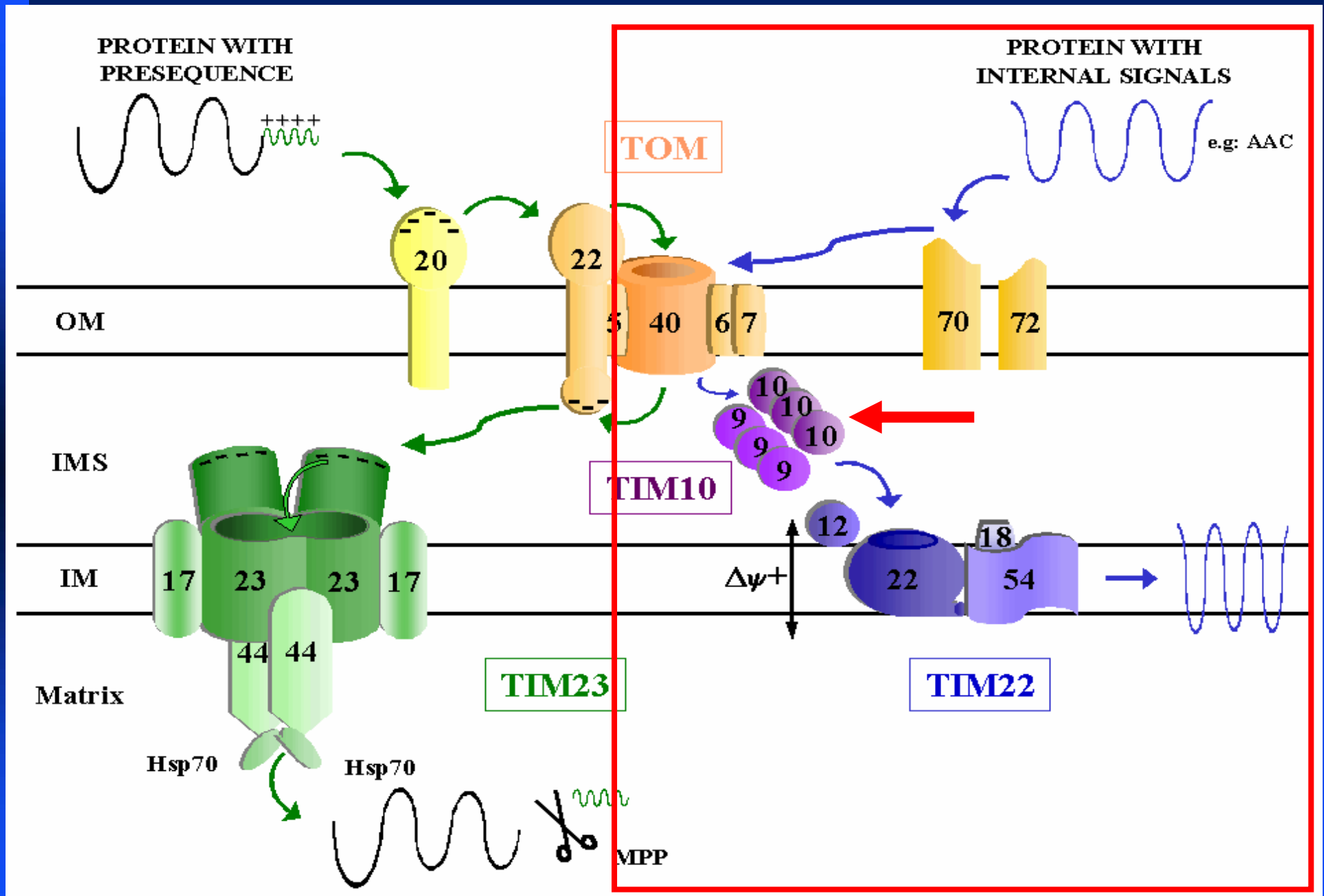
In mammalian cells mitochondrial carriers are involved in a number of disorders:

- diseases, like several myopathies
- obesity
- programmed cell death (apoptosis)


PROTEIN WITH PRESEQUENCE
++++
PROTEIN WITH INTERNAL SIGNALS
e.g: AAC
TOM
20
22
OM
5
40
6
7
70
72
10
10
9
10
9
9
IMS
TIM10
12
18
IM
17
23
23
17
Δψ+
22
54
44
44
Matrix
TIM23
TIM22
Hsp70
Hsp70
MPP

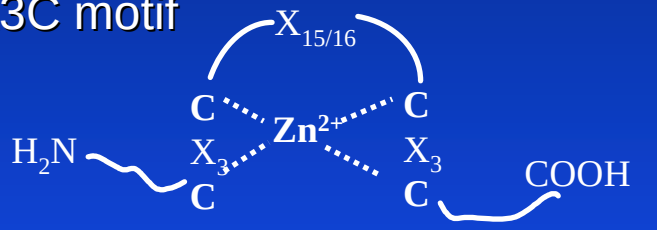
Distinct features of the “carrier pathway” compared to “matrix targeting”:

- 1. Requirement for a chaperone function in the IMS
- 2. No ATP hydrolysis in the matrix is necessary
- 3. No translocation motor in the matrix is required
- 4. The electrochemical membrane potential across the IM is enough to complete insertion at the IM

Properties of the “small Tim proteins” - 1

- **Sequence characteristics:**
  - They are all homologous
  - They are intrinsically soluble (No transmembrane domains)
  - They have a putative “zinc-binding” motif, twin CX3C motif
  - Found in all eukaryotic cells
  - Human homologue of Tim8

  involved in Mohr-Tranebjaerg syndrome



- **Organisation in assemblies:**
  - Tim9/10 and Tim8/13 are found in 70 kDa complexes in the IMS
  - Tim12 is associated with the membrane-embedded TIM22 complex (IM)

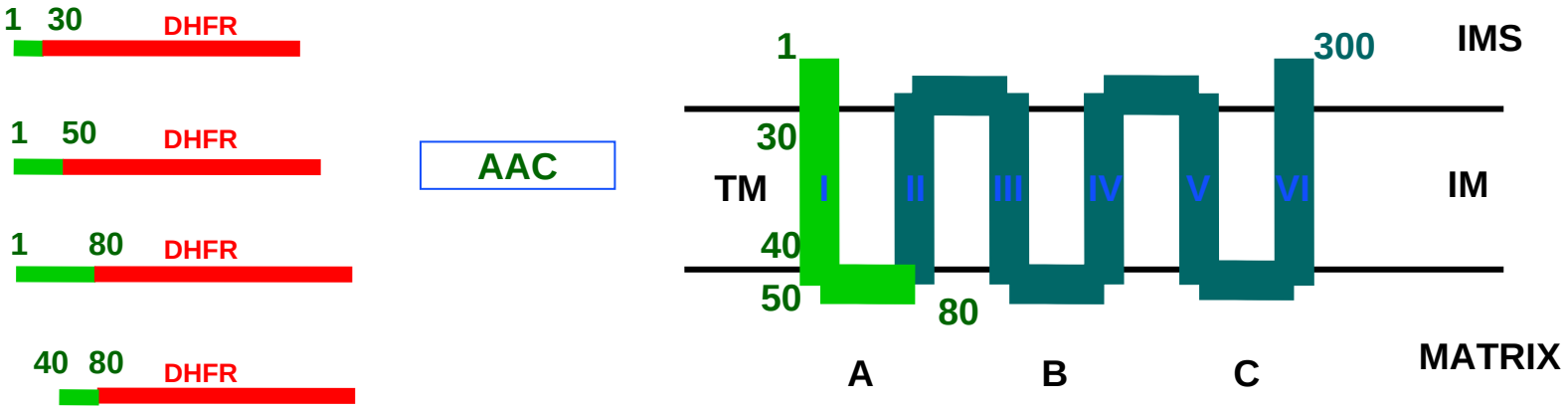
Properties of the “small Tim proteins” - 2

- **Function:**
  - Tim9/10 (essential) function as chaperones in the IMS and facilitate targeting to the IM
  - Tim12 (essential) facilitates insertion in the context of the TIM22 complex
  - Tim8/13 are non-essential but also seem to function as chaperones in the IMS

The AAC substrate: targeting signals??

Which are the sorting and insertion signals of the carrier proteins?

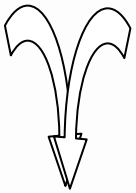
A. Deletion Analysis



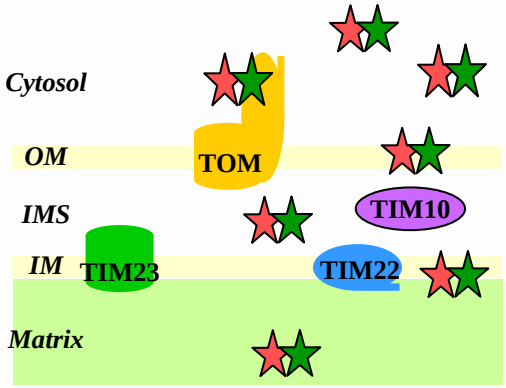
B. Site-directed mutagenesis

Radiolabelled protein synthesised in a rabbit reticulocyte lysate.

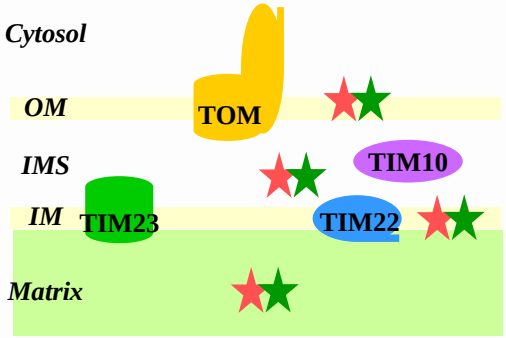
Import for 10min at 30°C



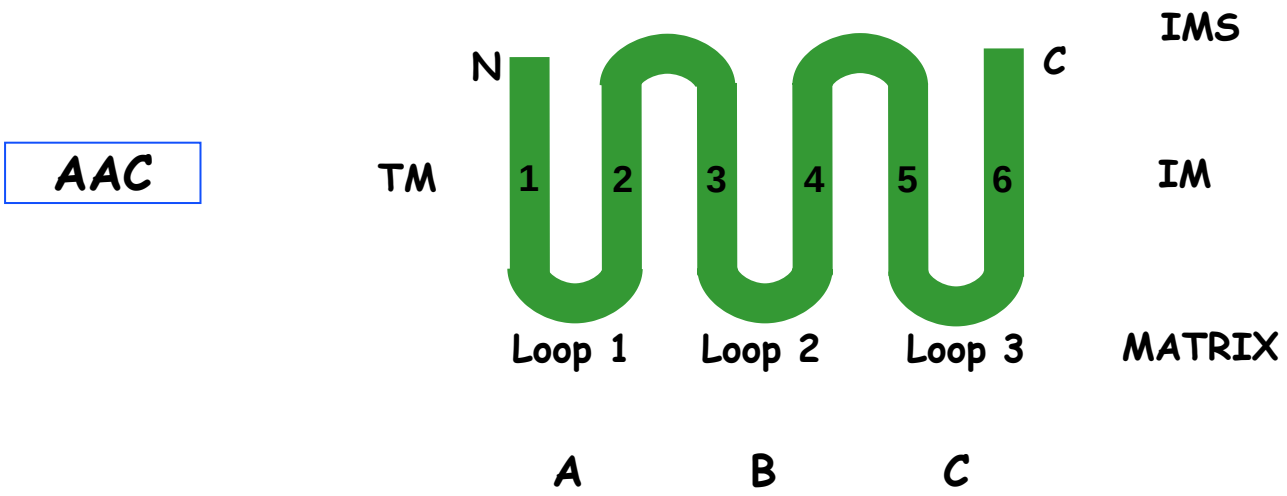
Purified mitochondria

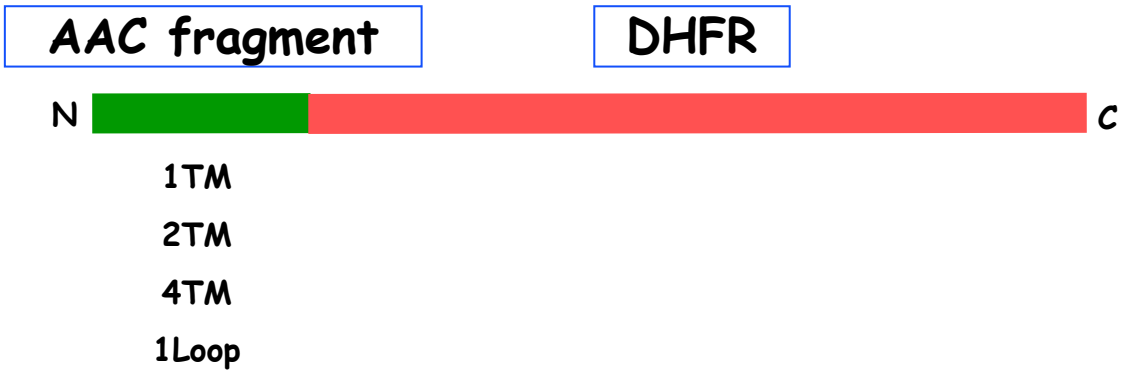


Proteinase K treatment

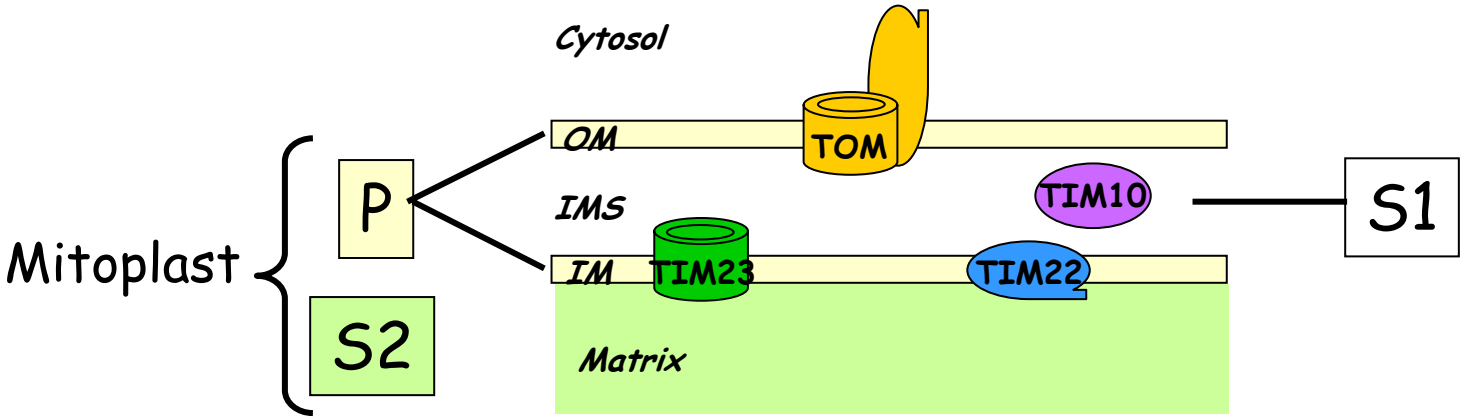


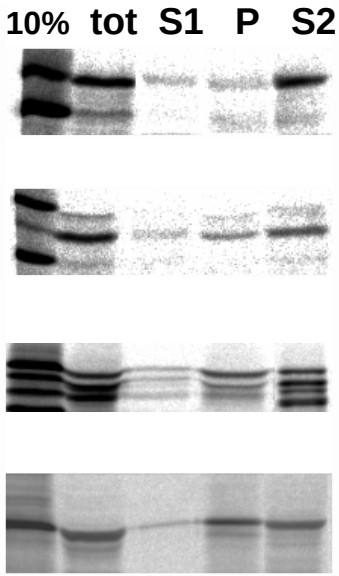
AAC-DHFR CONSTRUCTS





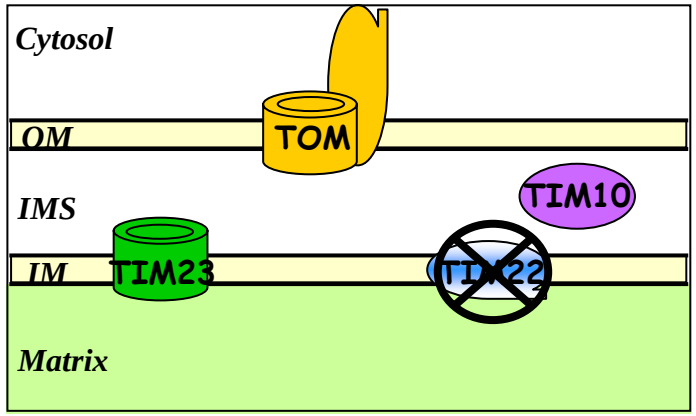
LOCALISATION





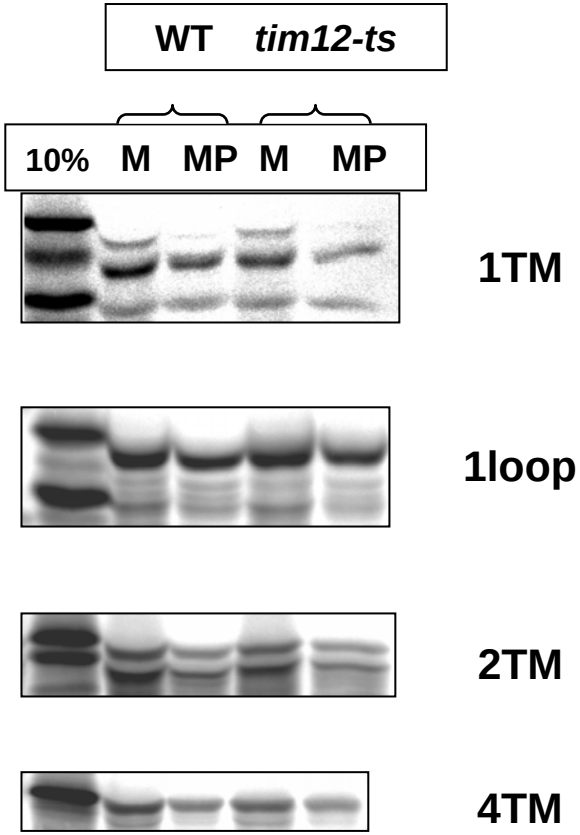
- Main localisation in IM
- Increase TM numbers → IM and Matrix

IS THE TIM22 COMPLEX REQUIRED FOR IMPORT?

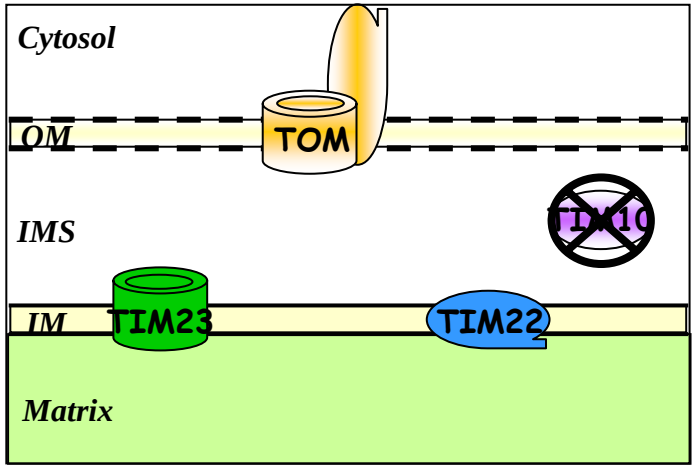


Import into *tim12-ts* mitochondria

- **No Tim12**
- **No Tim22**

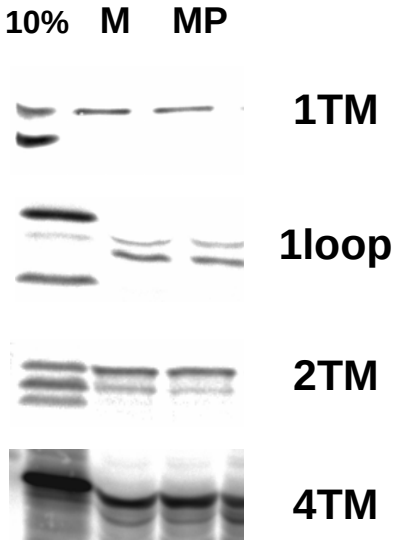


IS THE TIM10 COMPLEX REQUIRED FOR IMPORT?

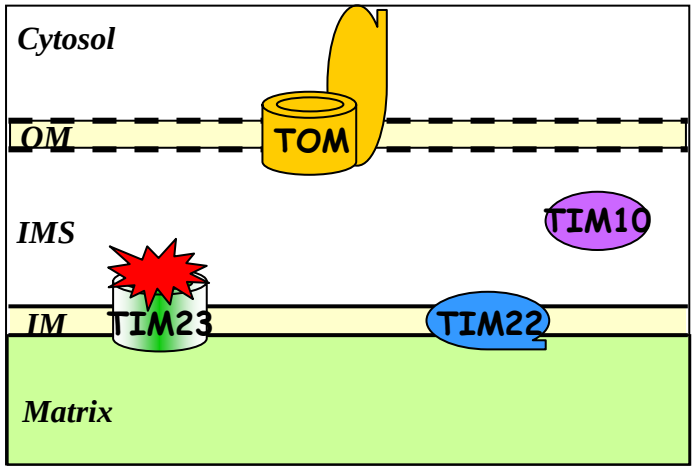


Import into Mitoplasts

- **No TIM10 complex**



IS THE TIM23 COMPLEX REQUIRED FOR IMPORT?

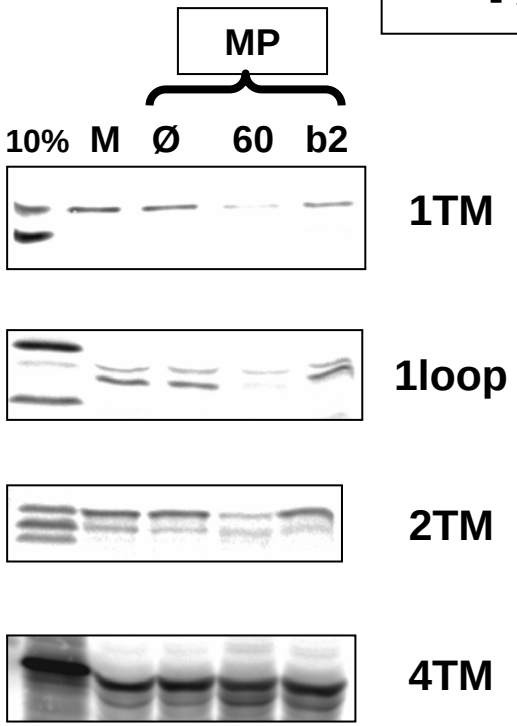


Import into mitoplasts +

peptide competition

-Hsp60= presequence peptide

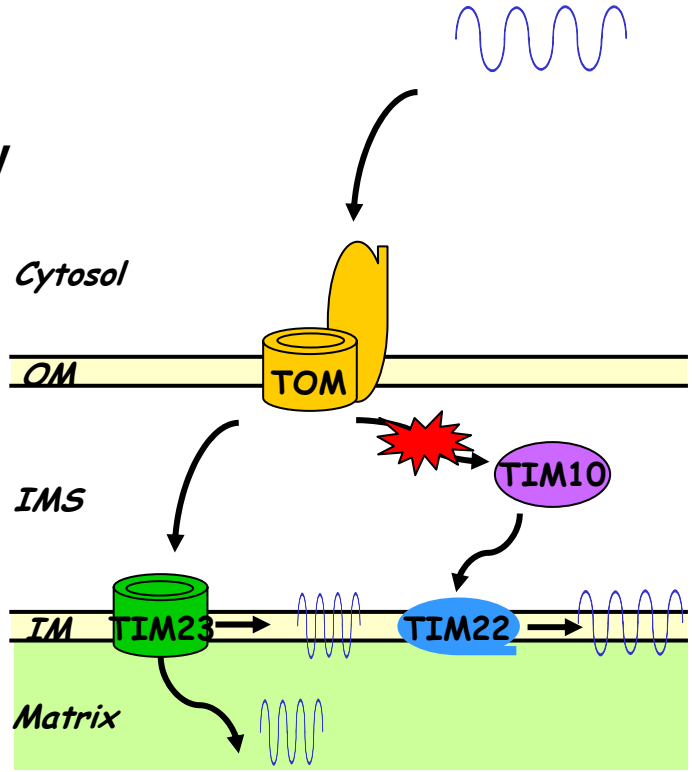
- Synb2= charged peptide



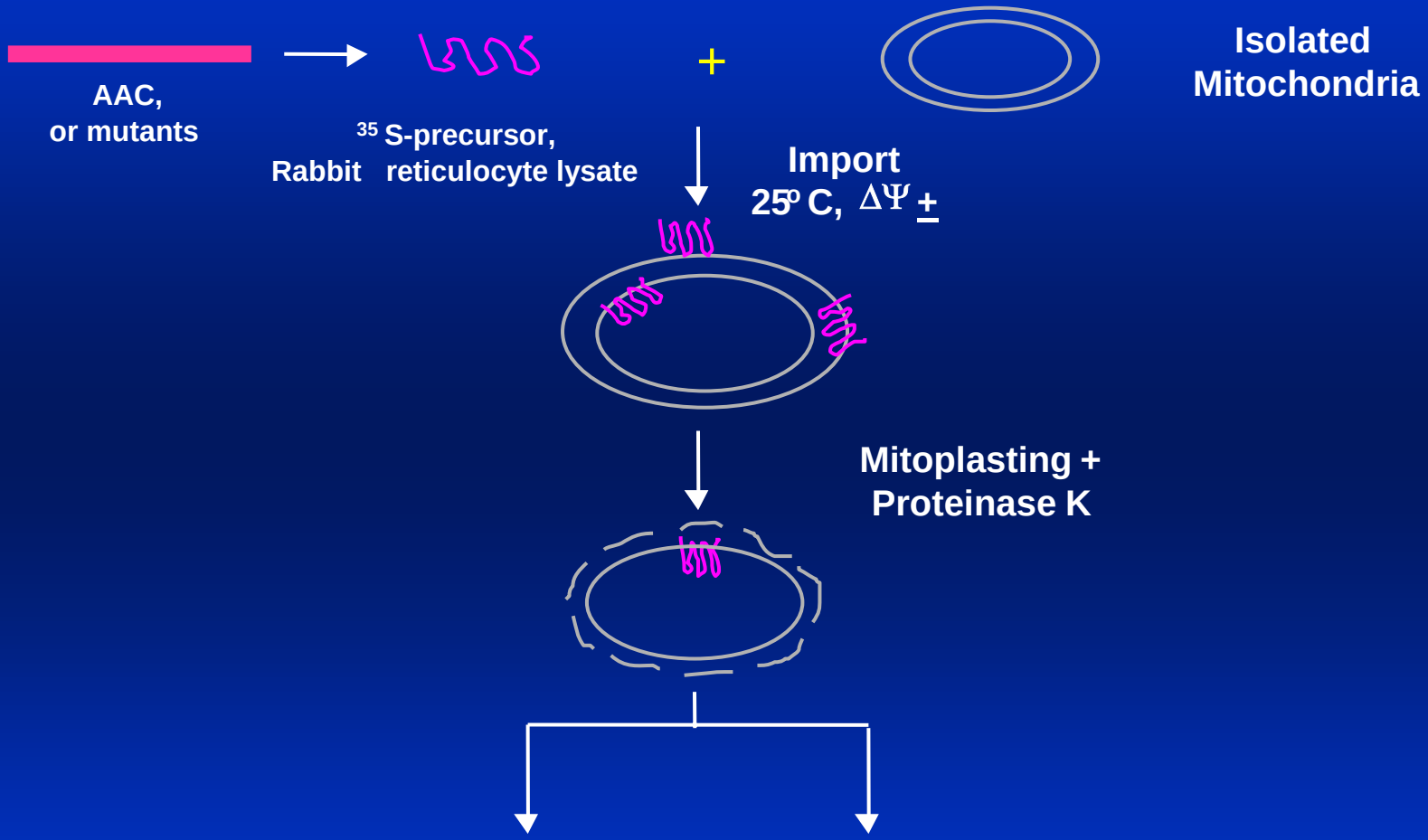
WORKING MODEL

Sufficient targeting information within each construct, BUT:

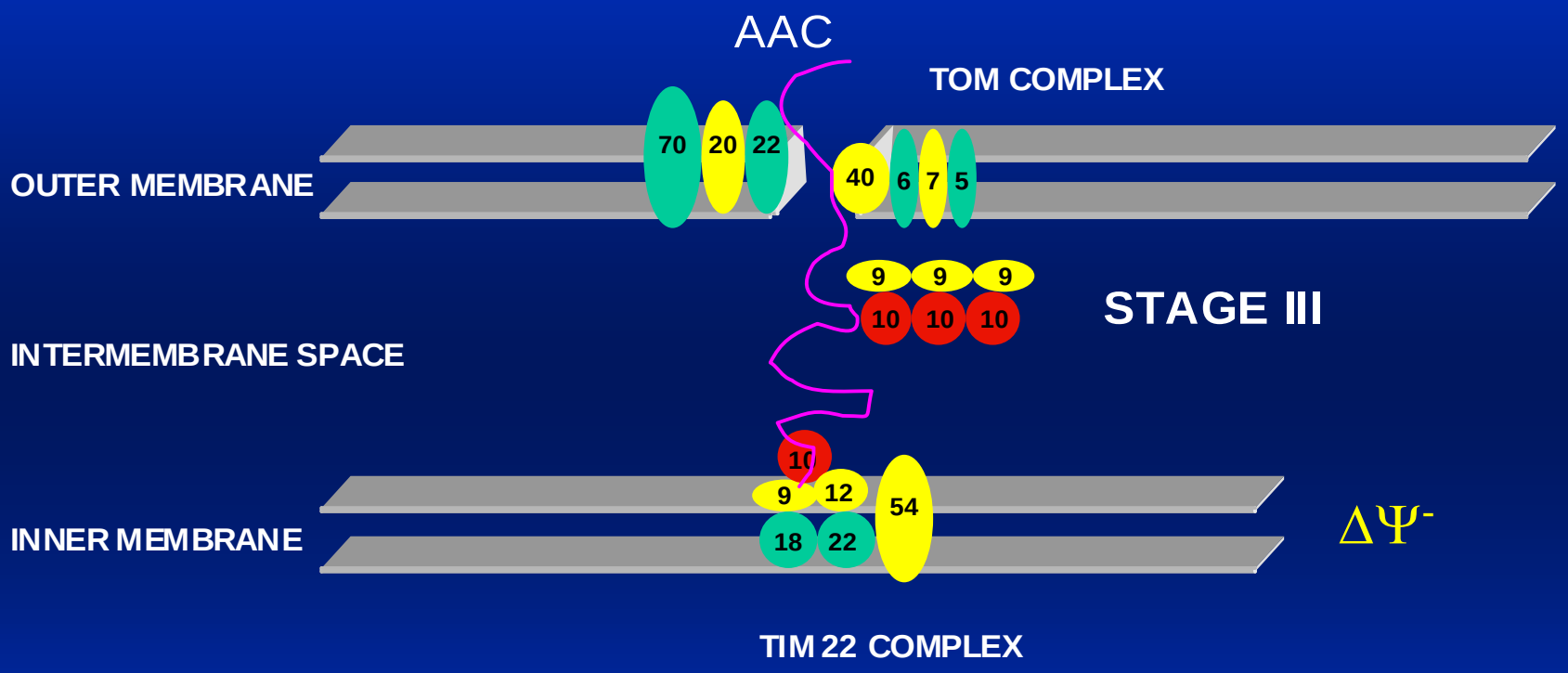
- **No interaction with TIM10 complex**
- **Therefore no targeting to TIM22**
- **Translocation via TIM23 as a default pathway**



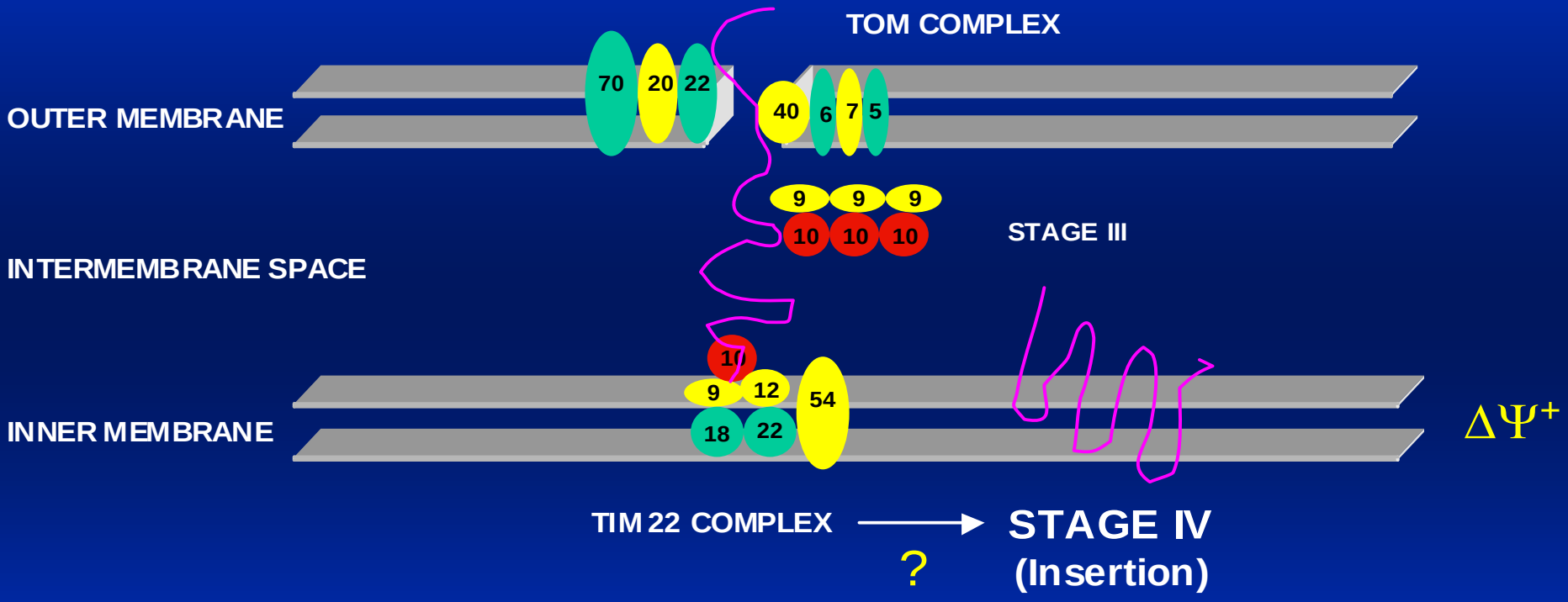
Import Scheme for AAC



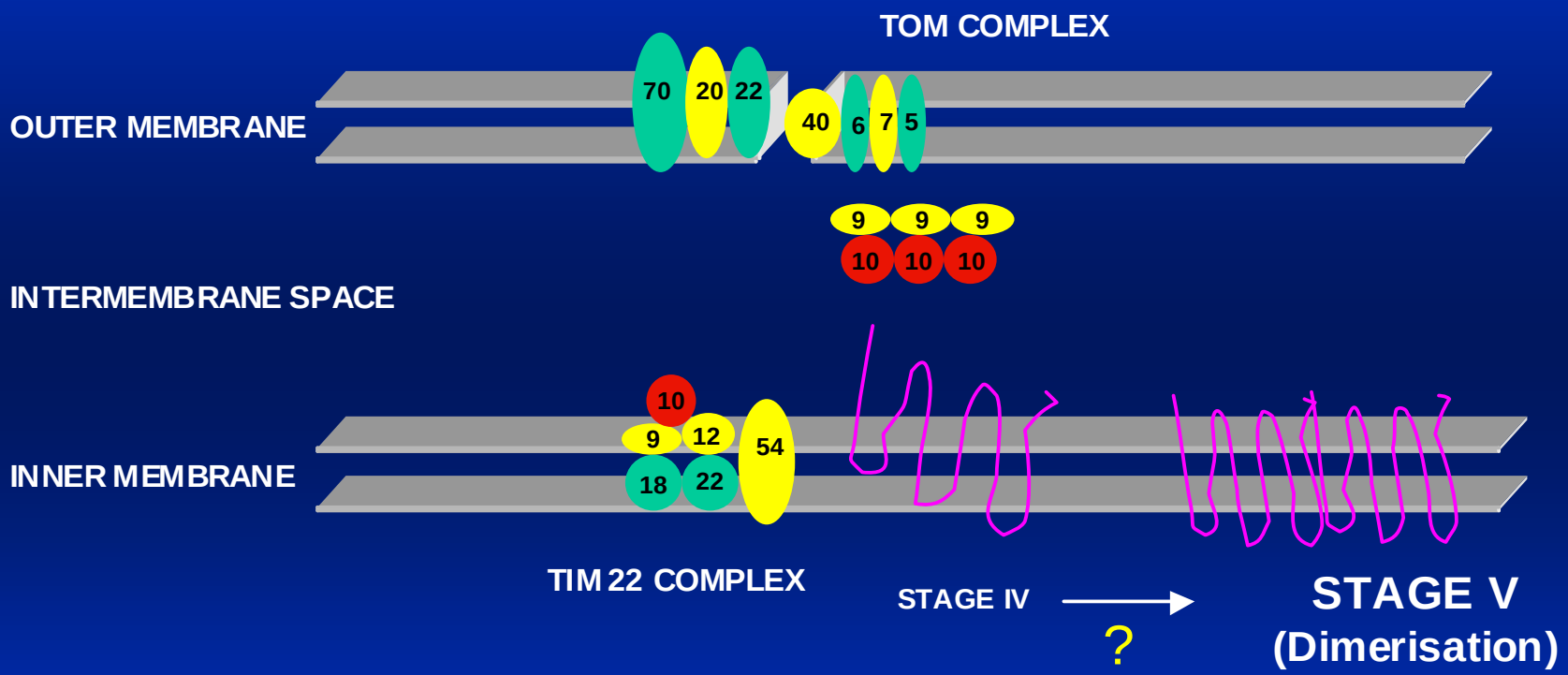
Stage III of AAC import

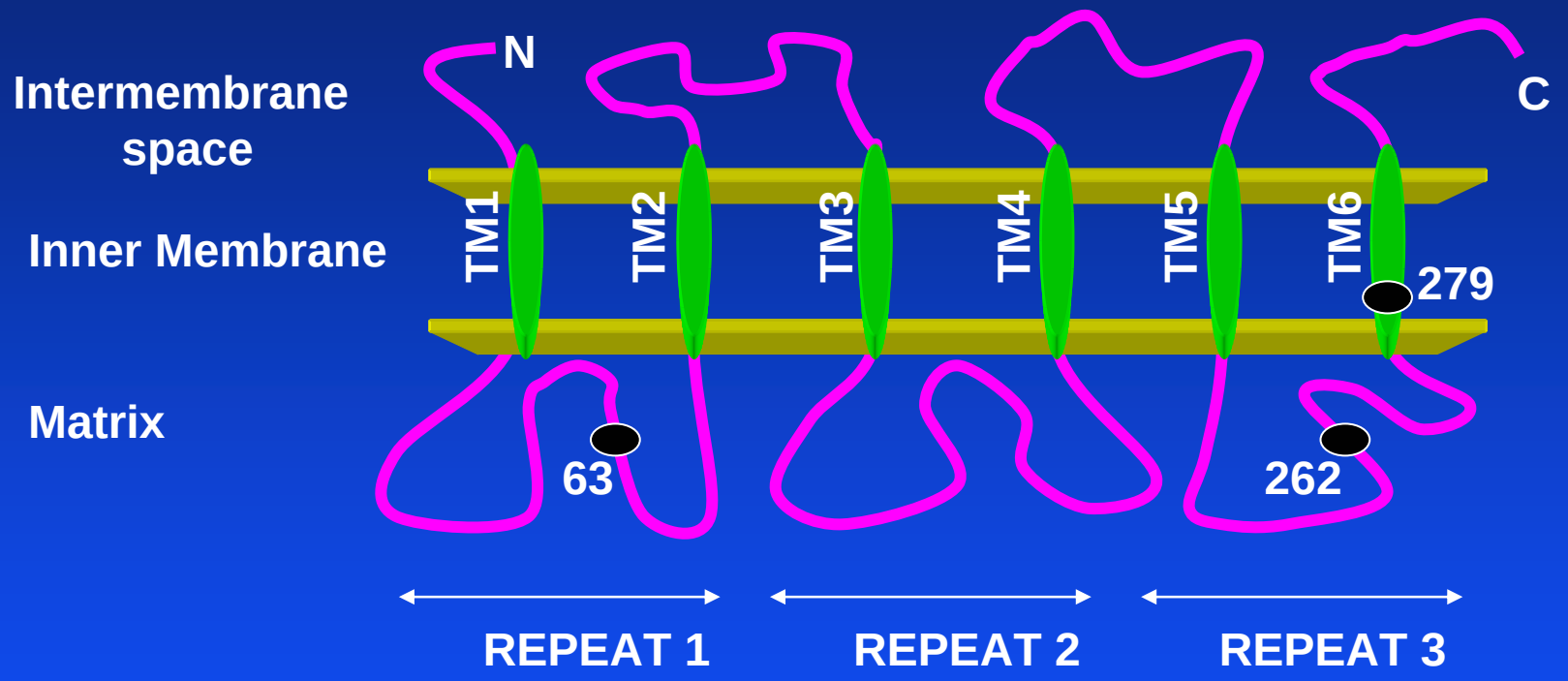


Stage IV of AAC Import



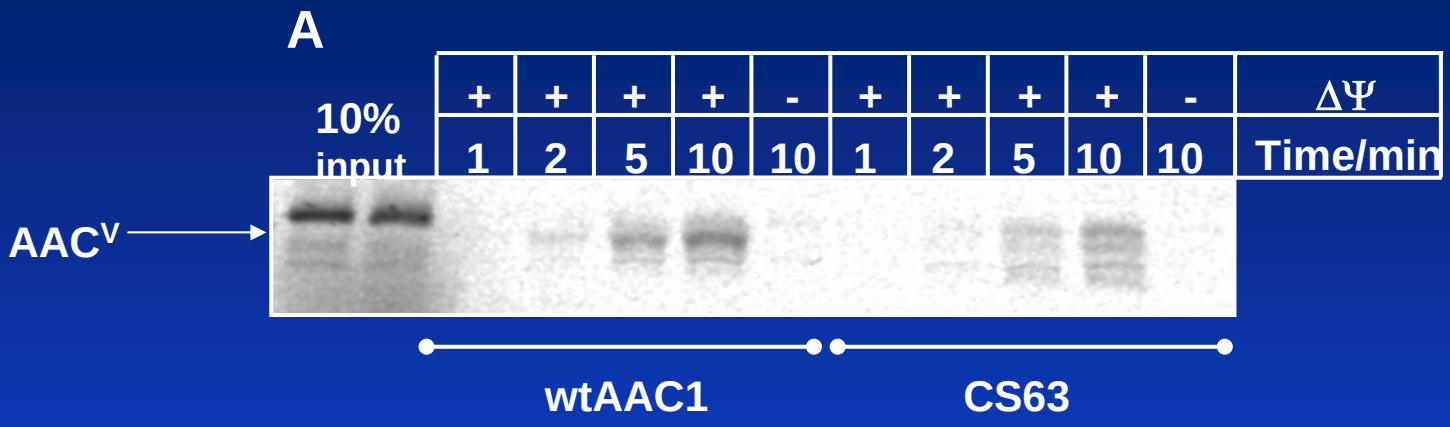
Stage V of AAC Import

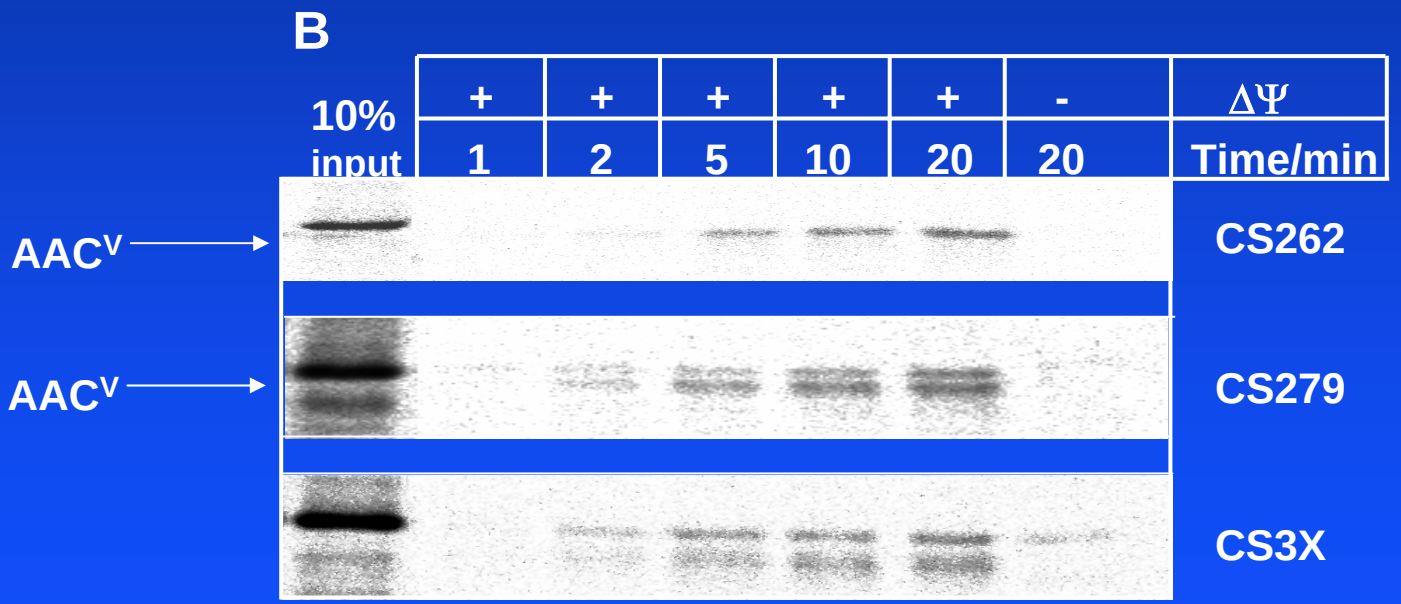




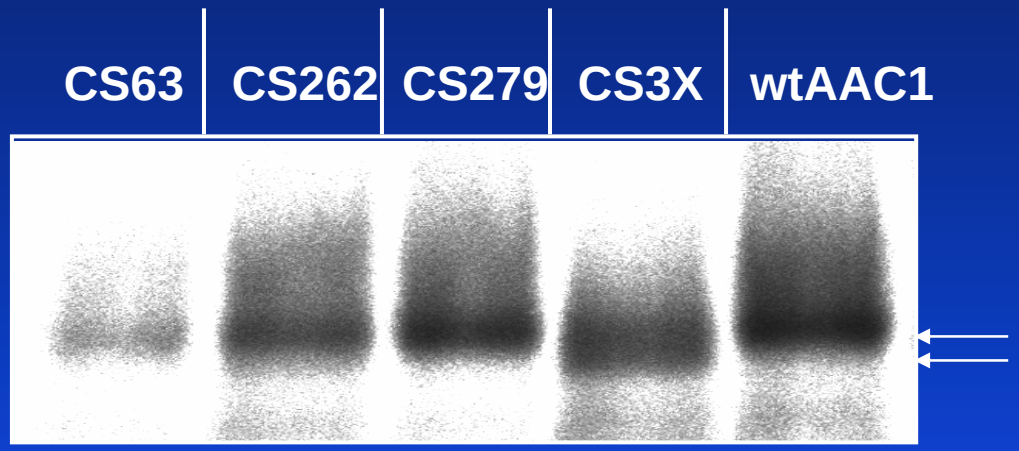
= Cysteine residue

Cys mutants affecting AAC insertion in the IM





Cys mutants affecting AAC insertion in the IM



- Functionally impaired Cys mutants have a significant defect also in insertion and dimerisation of AAC1
- CS63 (first loop) and the triple Cys mutant are abrogated in their efficiency to form a dimer in the membrane

Can we reconstitute *in vitro* the TIM10 complex in a functional form?

Reconstitution of the complex from the individual subunits

1. Co-expression of Tim9 and Tim10 in *E. coli* on a single plasmid in an operon
2. Expression and purification of the individual proteins separately
3. Purification of the authentic complex from yeast mitochondria

YES!

Tim9, Tim10 necessary and sufficient for assembly of TIM10 complex

Reconstituted complex indistinguishable from mitochondrial complex

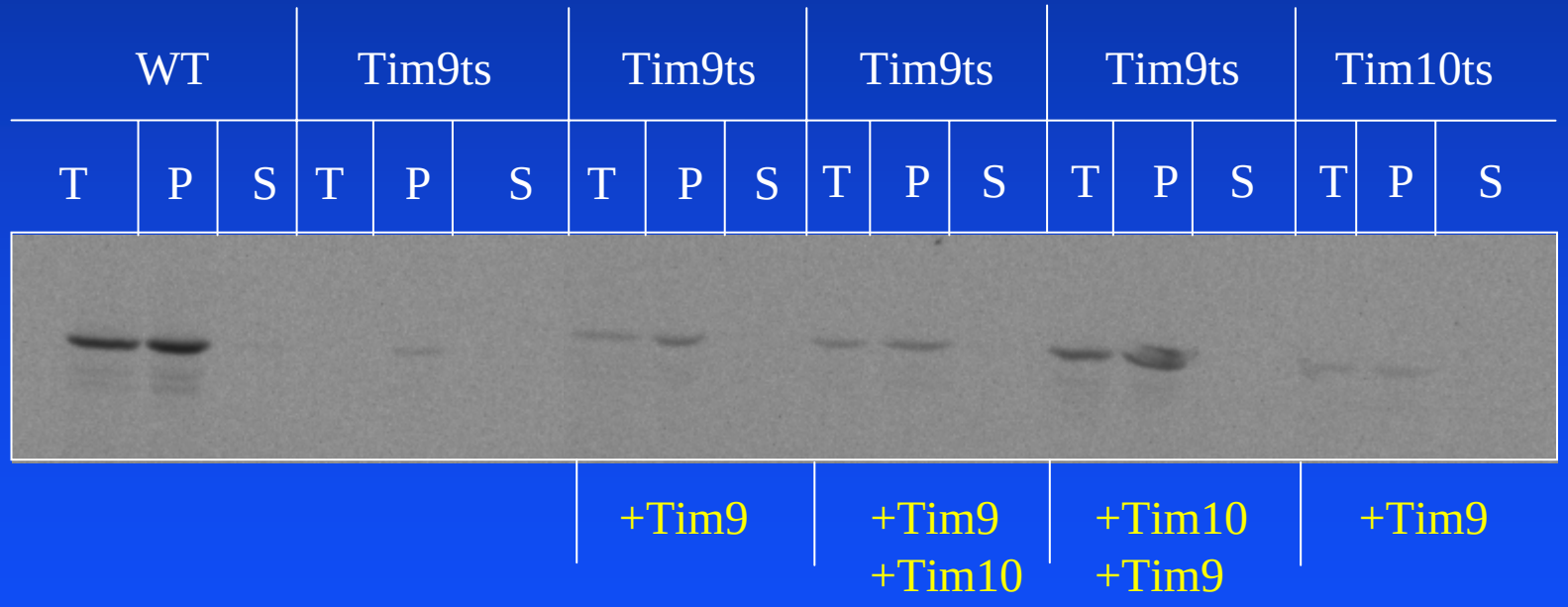
Is the reconstituted complex functional?

Functionality Assays:

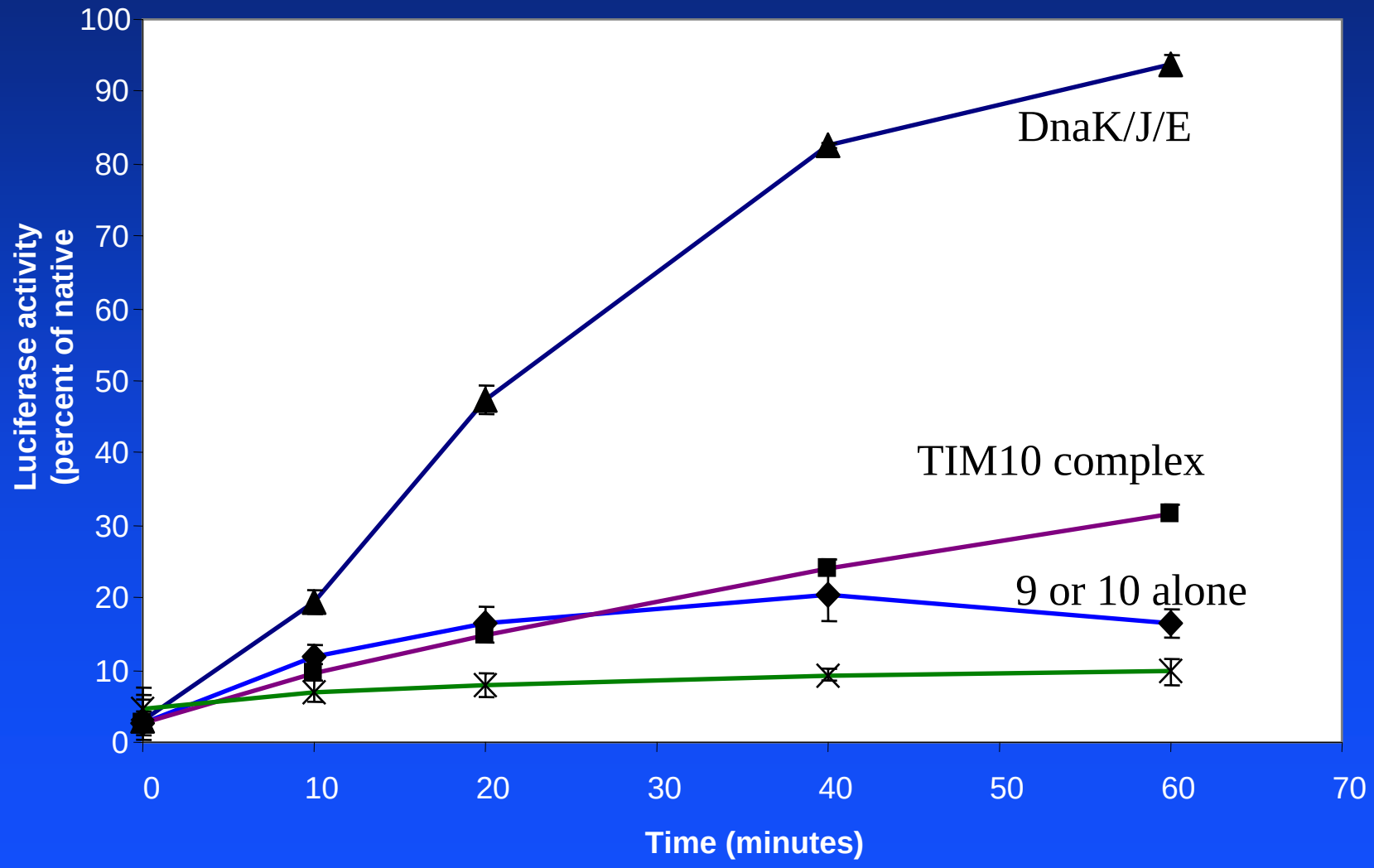
1. Restoration of AAC import into TIM10-depleted mitochondria
2. Binding in vitro to AAC
3. Chaperone activity in vitro

Reconstitution in Tim9ts Mitochondria

AAC import into Tim9ts Mitochondria
is restored after Import of Tim9 and/or Tim10

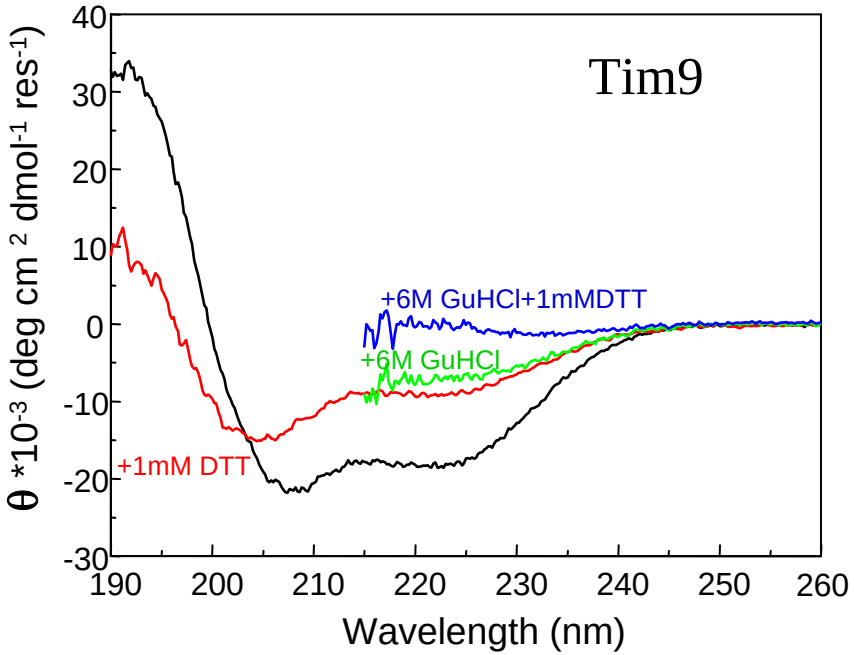


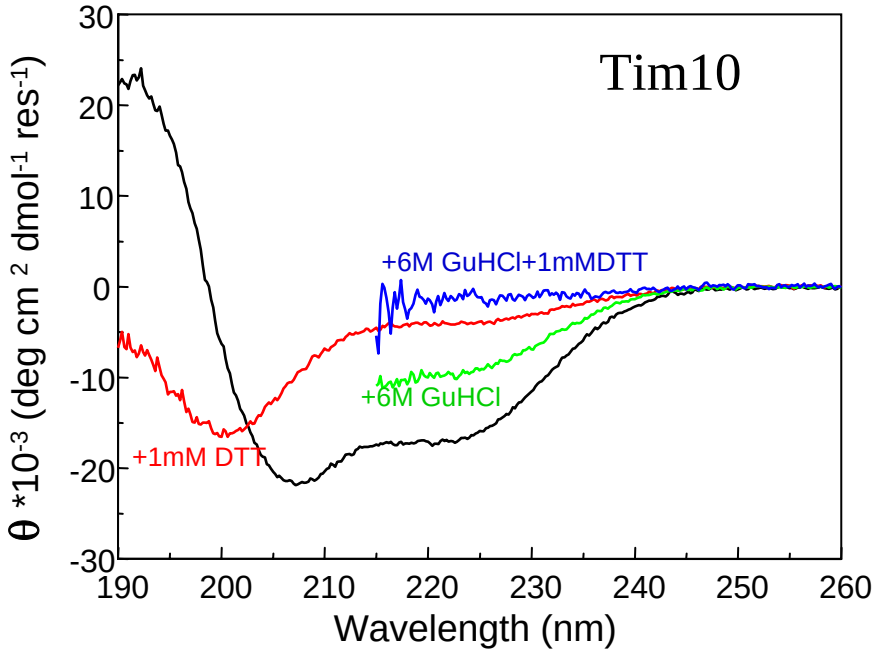
The TIM10 complex chaperones luciferase refolding



What is the structural basis for the assembly of the TIM10 complex?

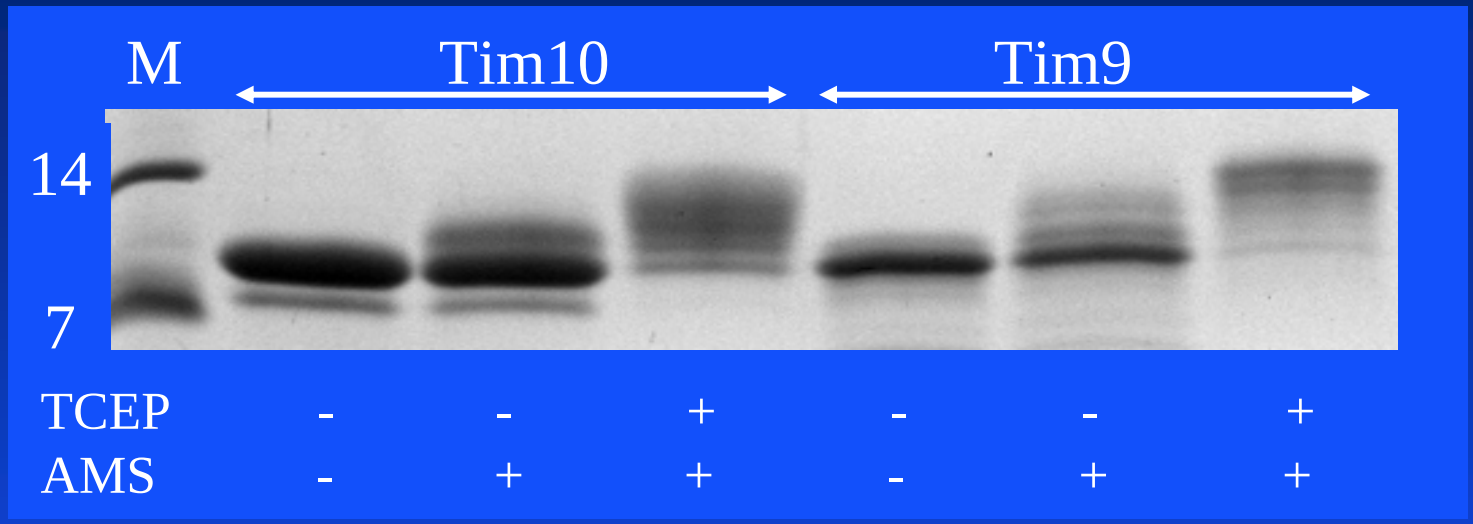
CD Analysis of individual Tim9 and Tim10





	α-helix	β-sheet	rest
oxTim10	65%	-	35%
red Tim10	8%	33%	59%
oxTim9	53%	8%	38%
redTim9	12%	39%	49%

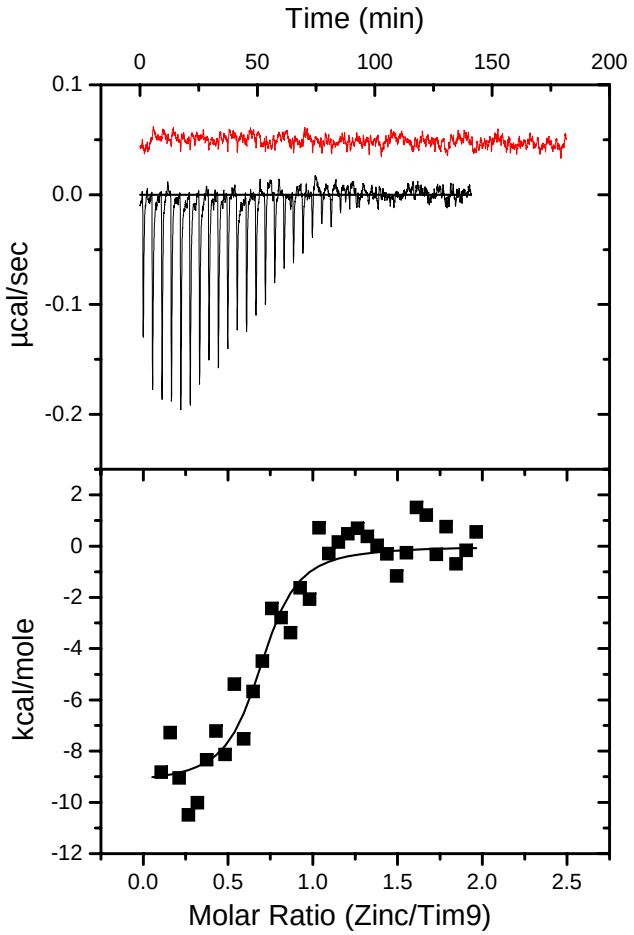
Thiol-trapping and accessibility by AMS and DTNB

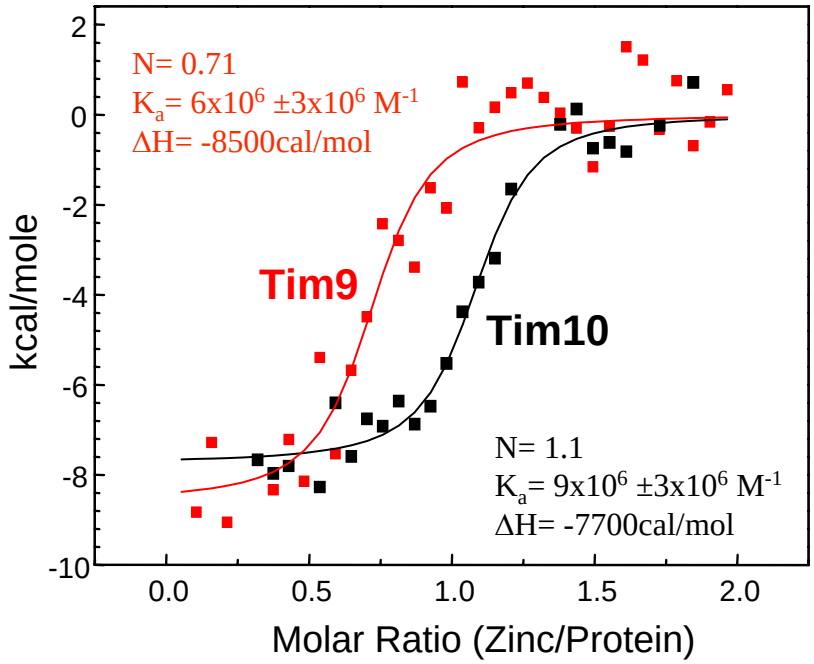


DTNB assay

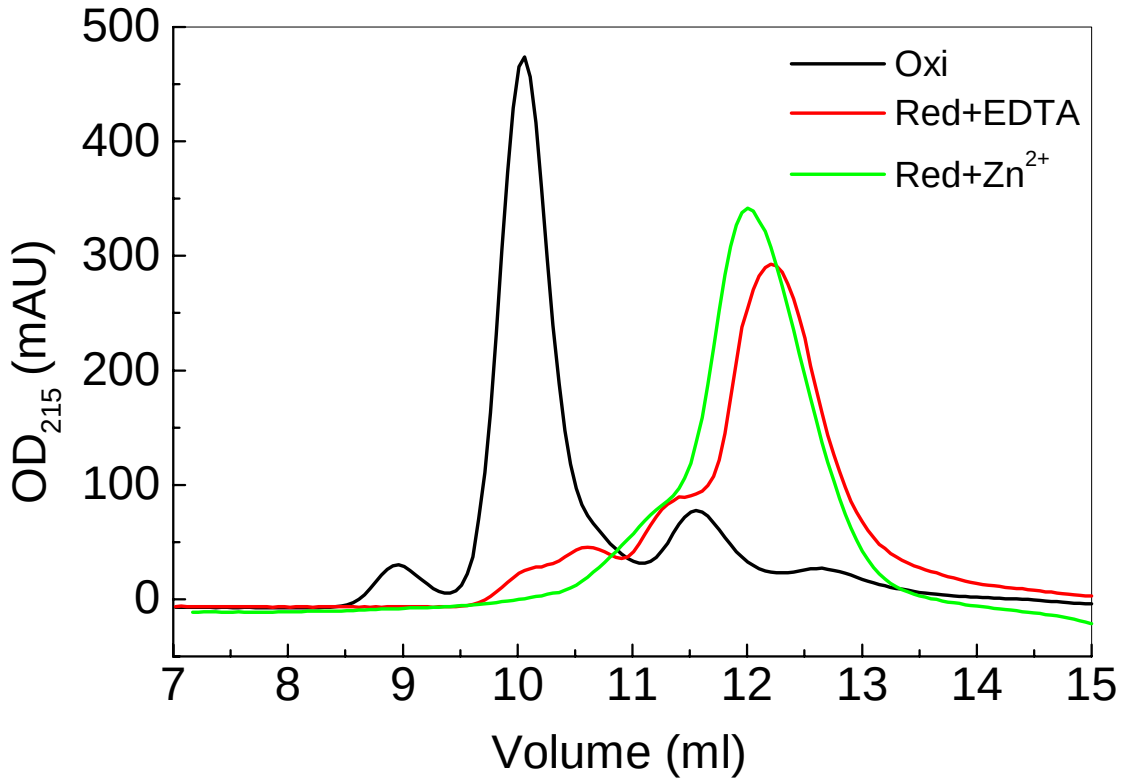
	GuHCl-DTT	GuHCl+DTT
Tim9	0	4.2 ± 0.5
Tim10	0	3.6 ± 0.3

Zinc binds to reduced Tim9 and reduced Tim10, not the oxidised states

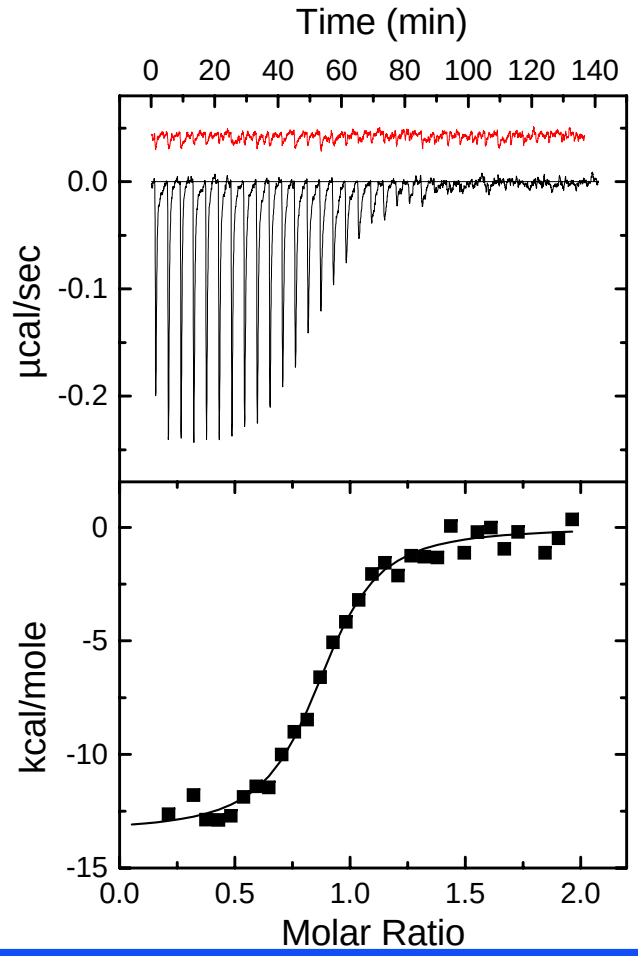




Oxidised Tim9 and oxidised Tim10 form the complex



ITC study of the interaction between Tim9 and Tim10

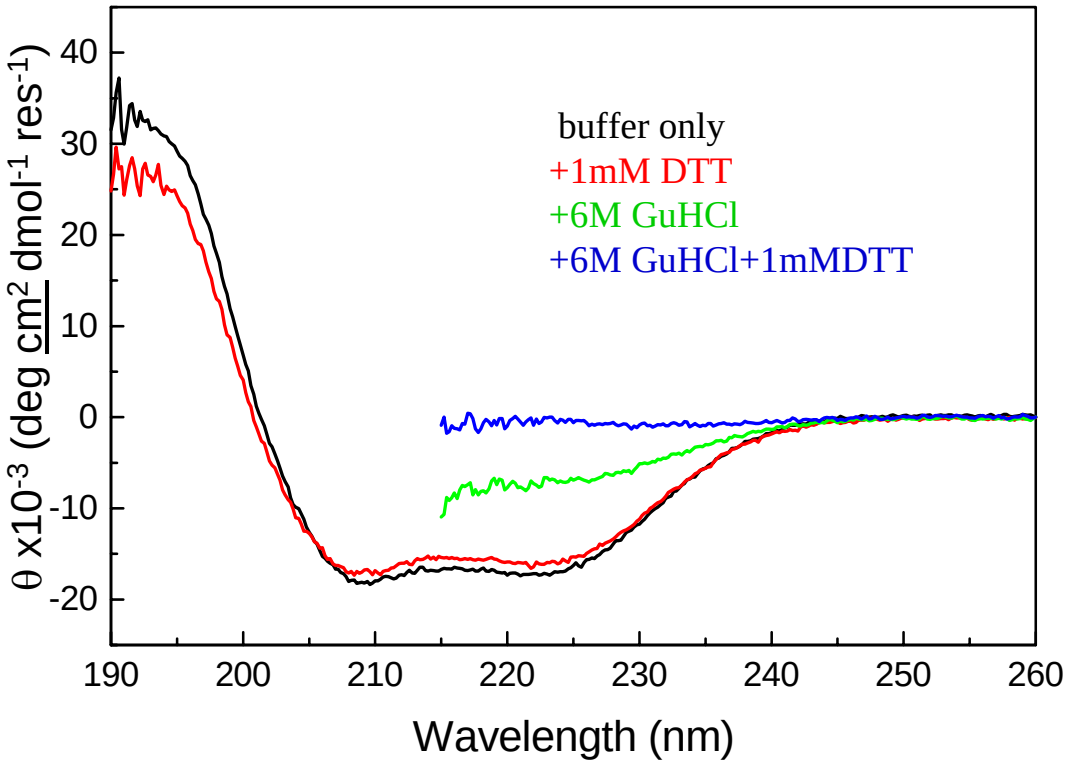


Reduced Tim9/Tim10

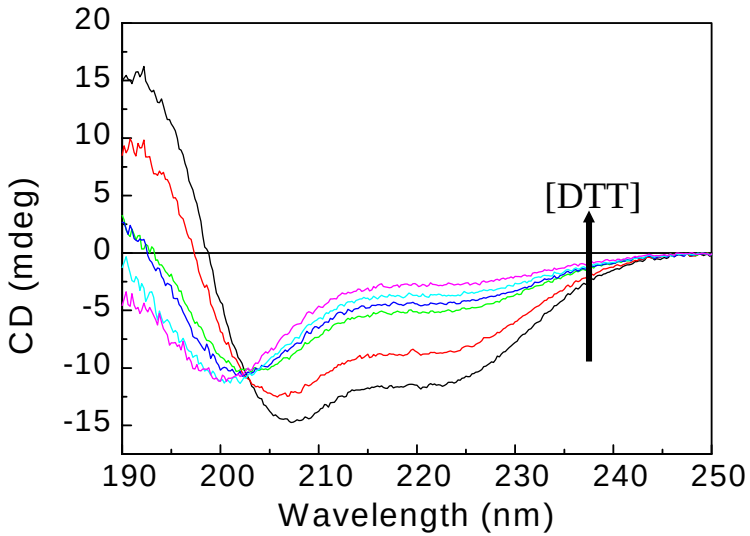
Oxidised Tim9/Tim10

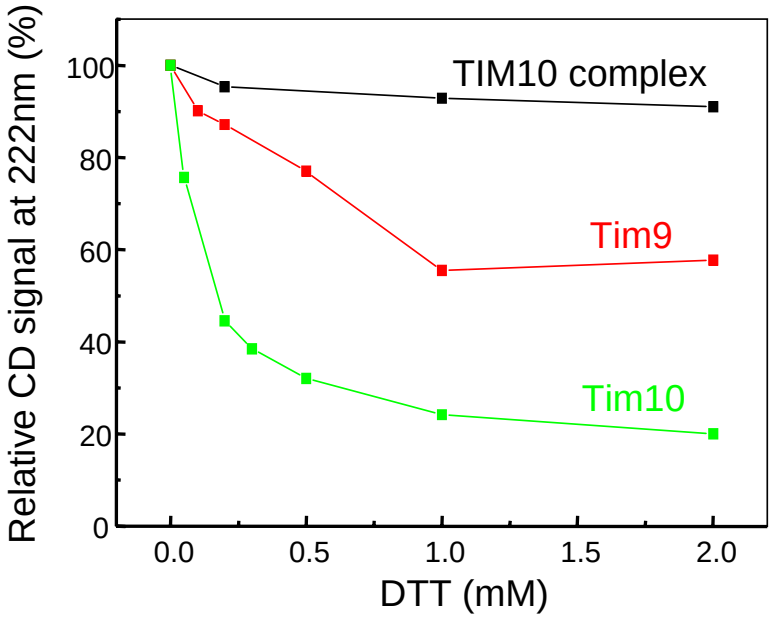
N= 0.9 (Tim10/Tim9)
$K_a = 5x10^6\ M^{-1}$
ΔH= -13kcal/mol

CD analysis of the TIM10 complex

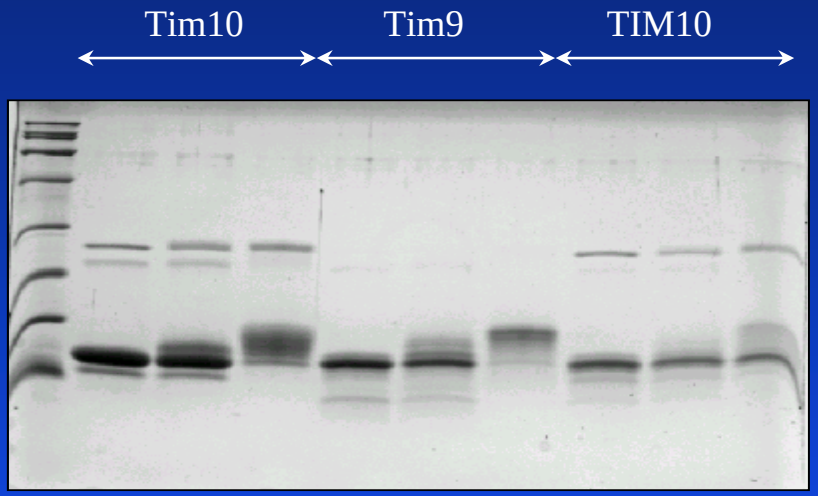


DTT concentration dependence



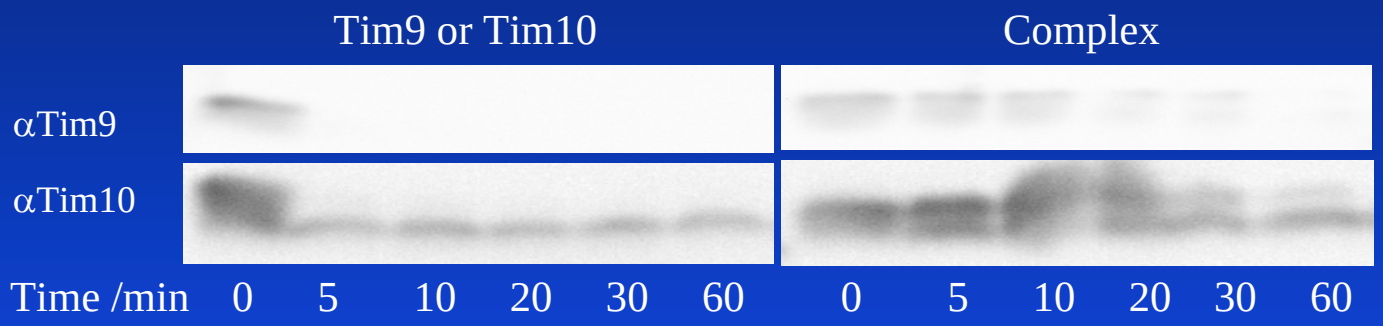


AMS and DTNB assay for TIM10 complex

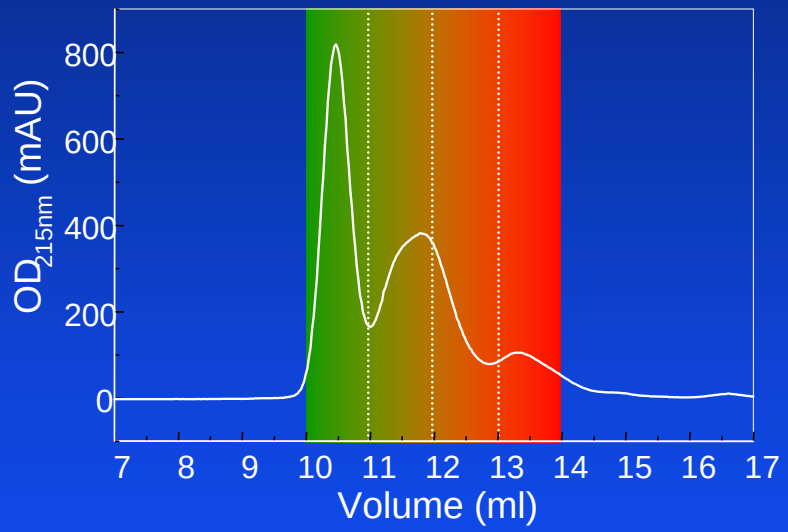


DTNB assay of the complex:
GuHCl-DTT: 0
GuHCl+DTT: 8.2±0.5 per Tim9/10

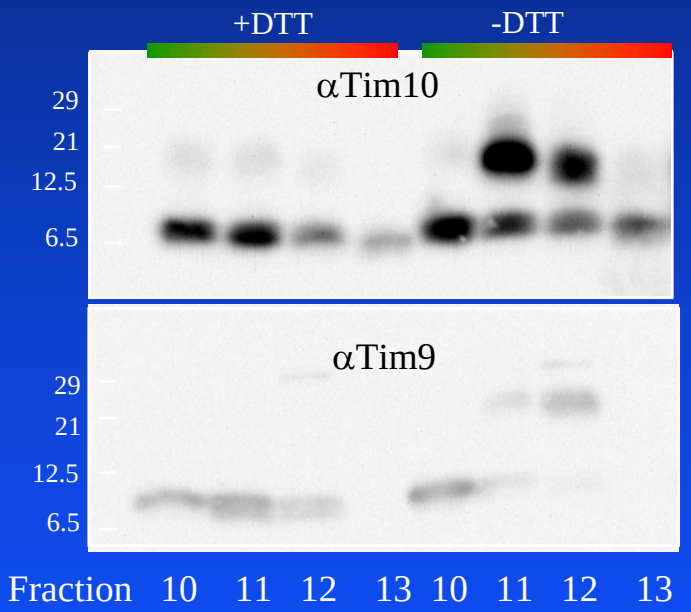
The TIM10 complex is more stable against trypsin digestion than the individual proteins



Inter- vs. *intra*- molecular disulfides: Misfolding & complex formation

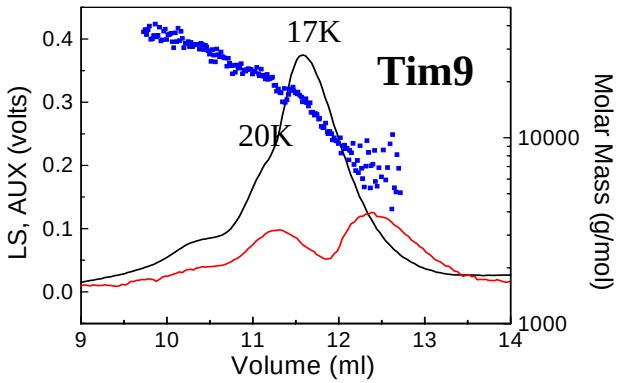


Western Blotting

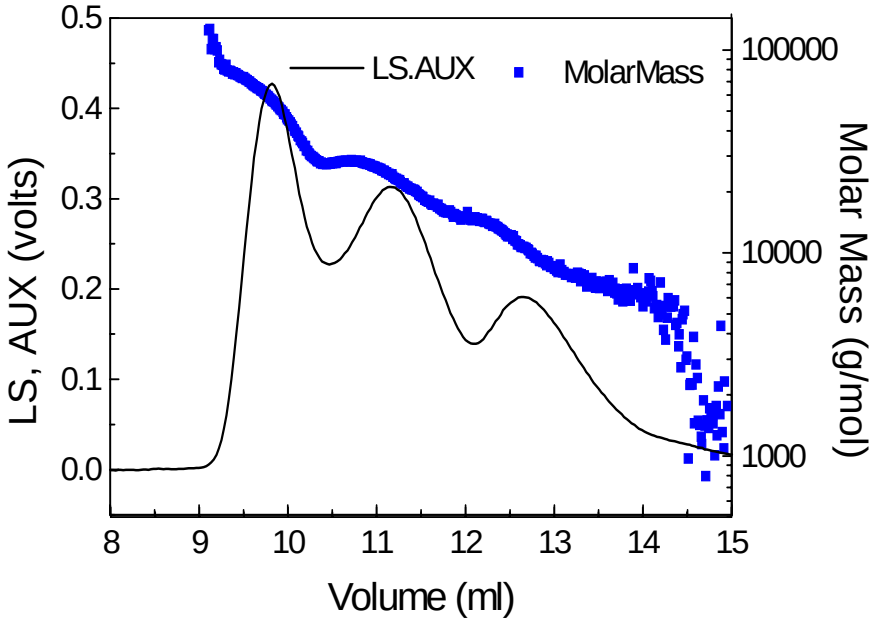


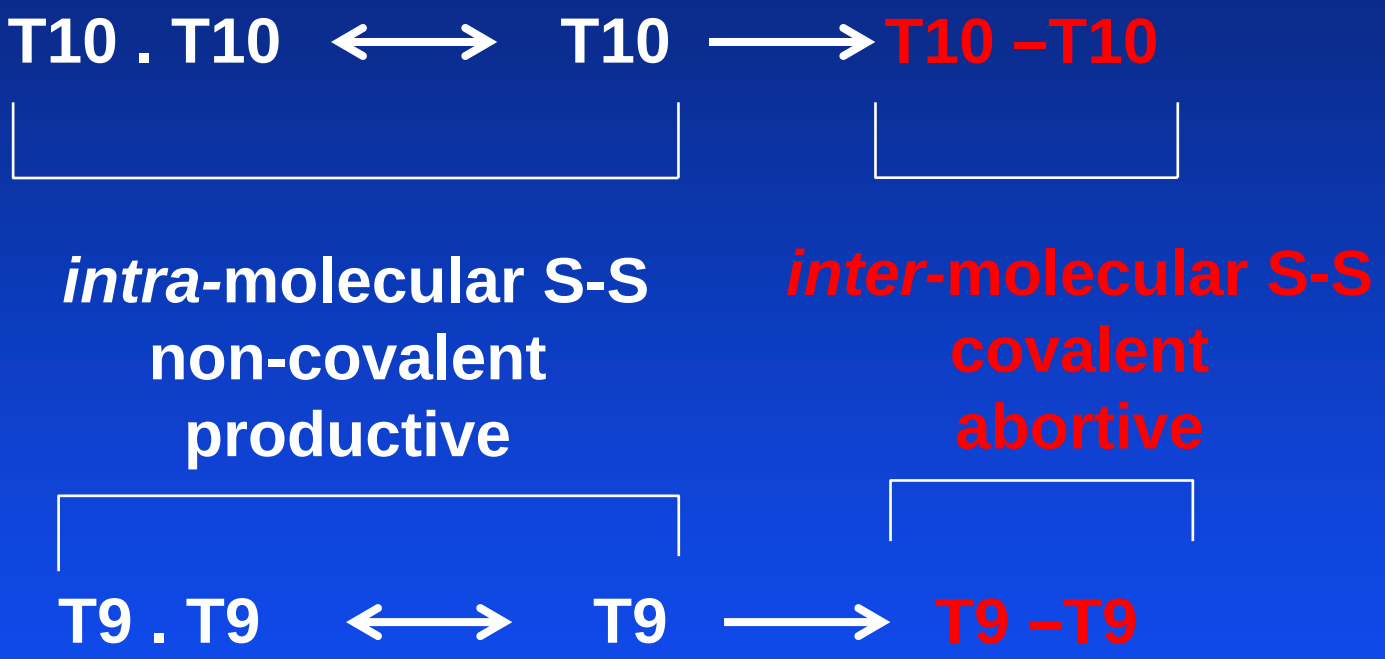
Dynamics and size analysis by multi-angle Light scattering

Individual proteins



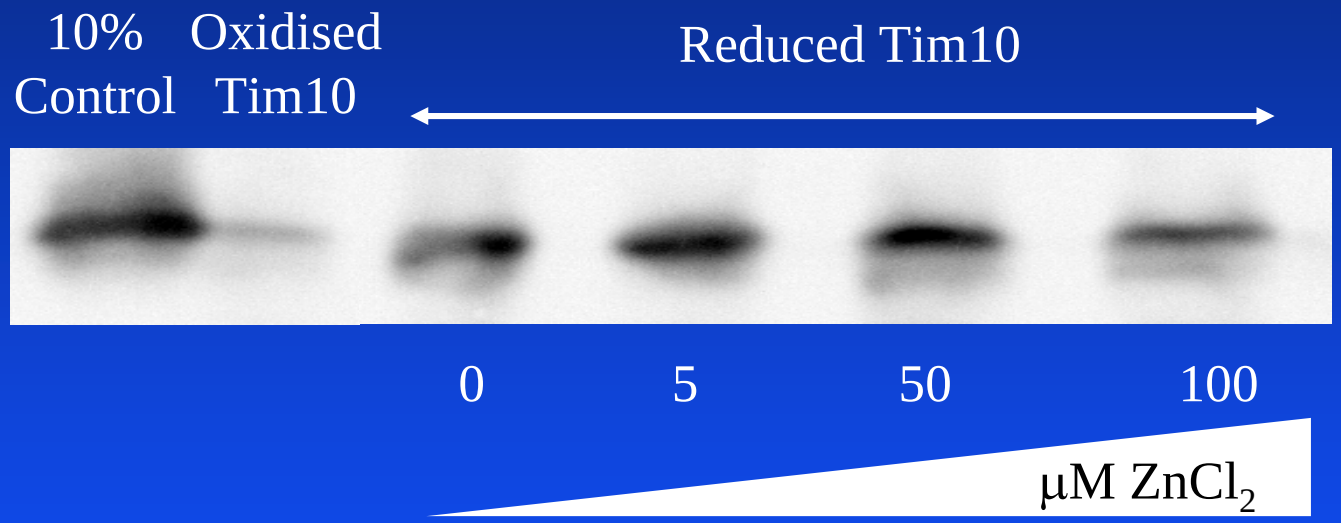
TIM10 complex




T10 . T10
T10
T10 –T10
intra-molecular S-S
non-covalent
productive
inter-molecular S-S
covalent
abortive
T9 . T9
T9
T9 –T9

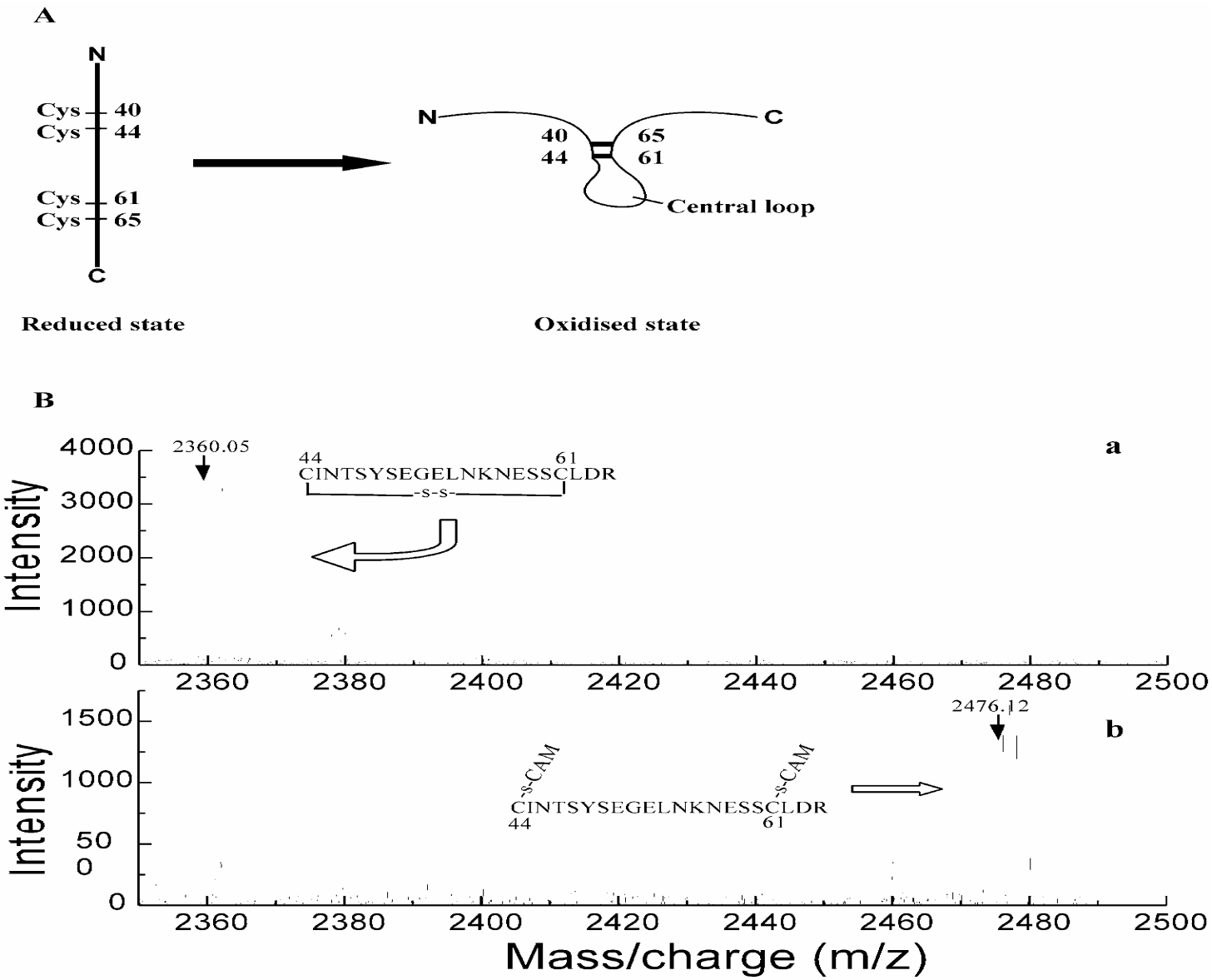
Compartment-specific redox regulation of TIM10 assembly?

Prior Oxidation inhibits import

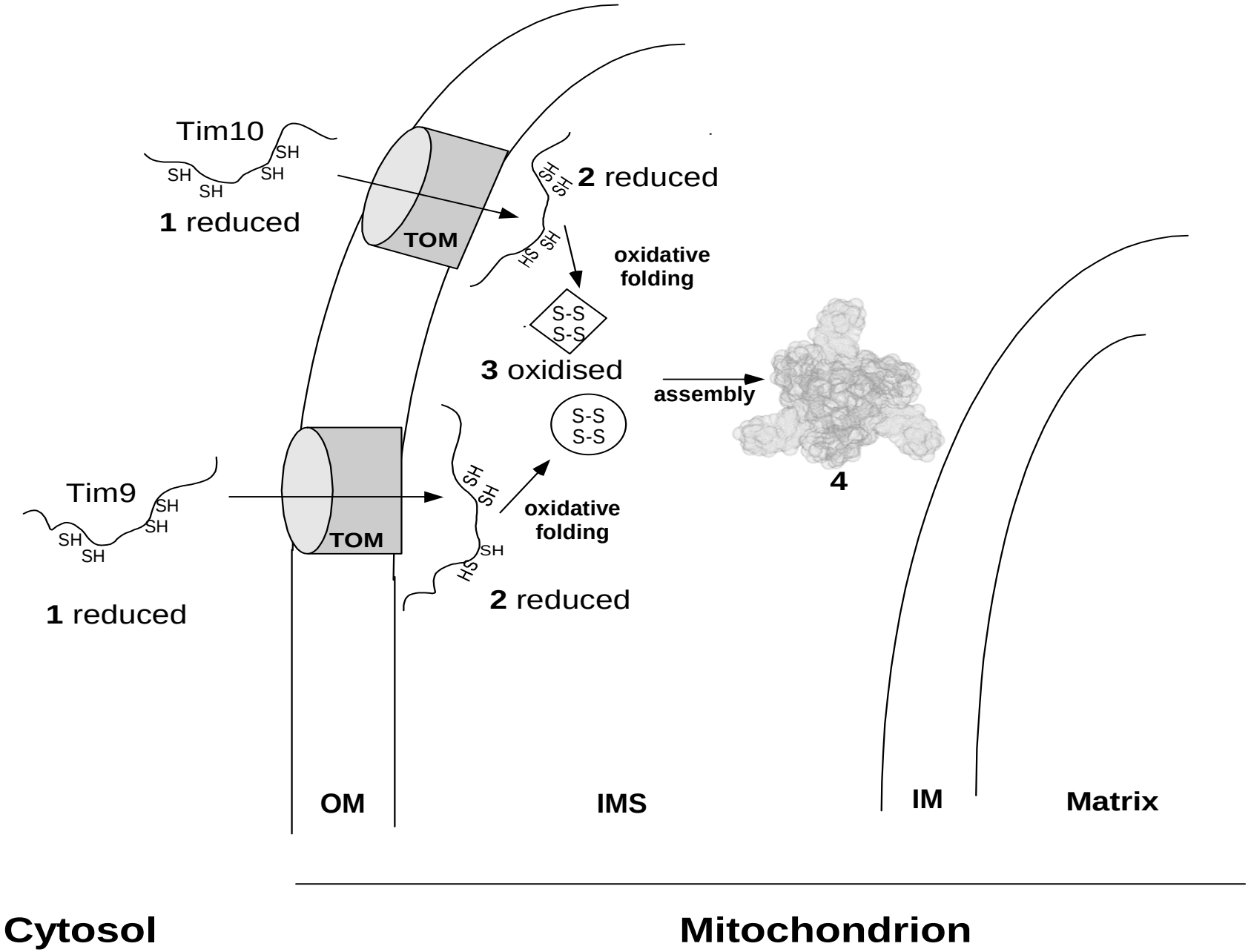


Additional data:

1. NEM alkylation
2. Cys mutants


A
N
Cys 40
Cys 44
Cys 61
Cys 65
C
N
C
40
44
65
61
Central loop
Reduced state
Oxidised state
B
a
2360.05
44
61
CINTSYSEGELNKNESSCLDR
-s-s-
Intensity
4000
3000
2000
1000
0
2360
2380
2400
2420
2440
2460
2480
2500
b
2476.12
s-CAM
s-CAM
CINTSYSEGELNKNESSCLDR
44
61
Intensity
1500
1000
50
0
Mass/charge (m/z)

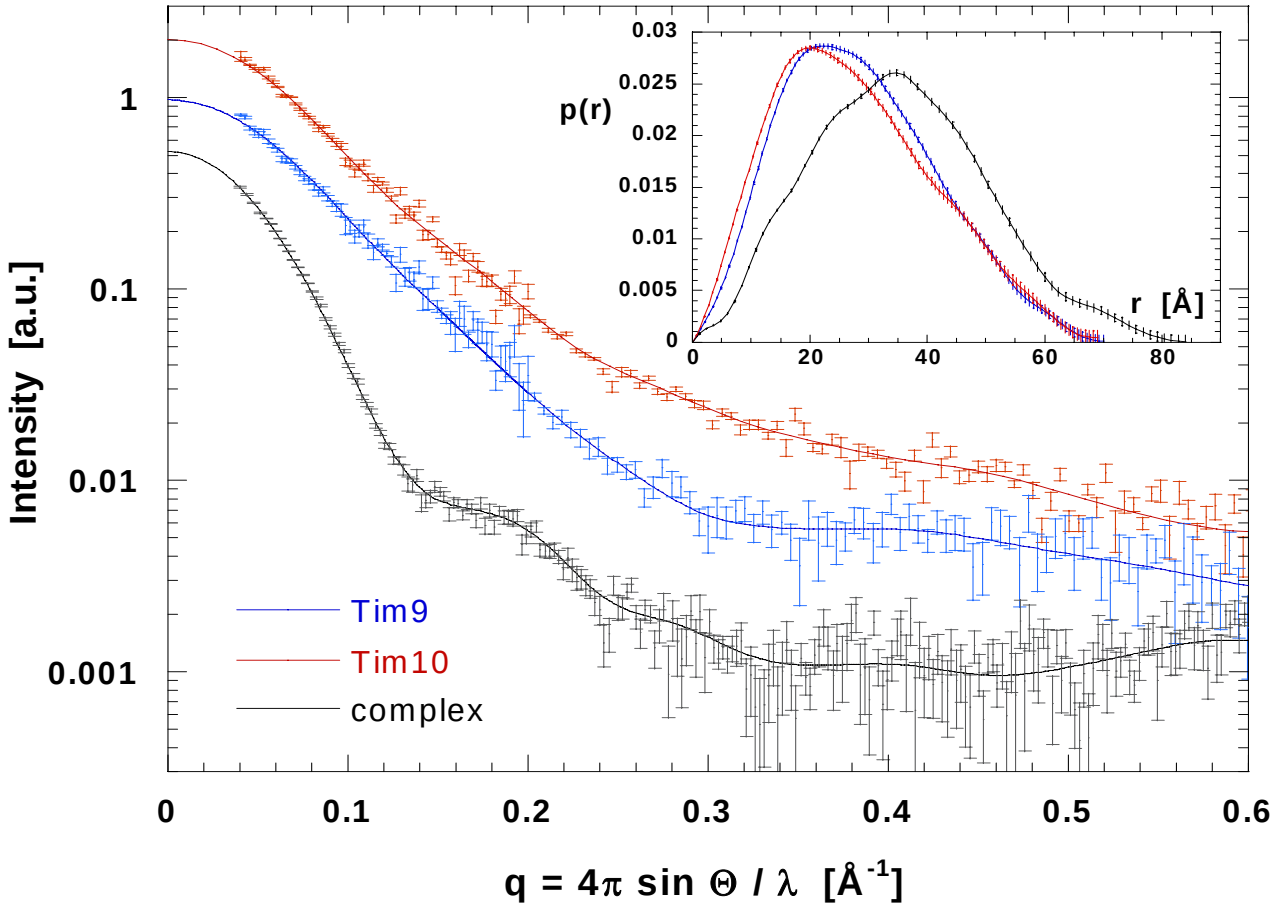
Oxidative folding locks the assembly of the TIM10 complex in the intermembrane space


Tim10
SH
SH
SH
SH
1 reduced
TOM
2 reduced
oxidative
folding
S-S
S-S
3 oxidised
assembly
4
Tim9
SH
SH
SH
SH
1 reduced
TOM
oxidative
folding
S-S
S-S
2 reduced
OM
IMS
IM
Matrix
Cytosol
Mitochondrion

Are there distinct functional domains in the small Tims?

3D structure?

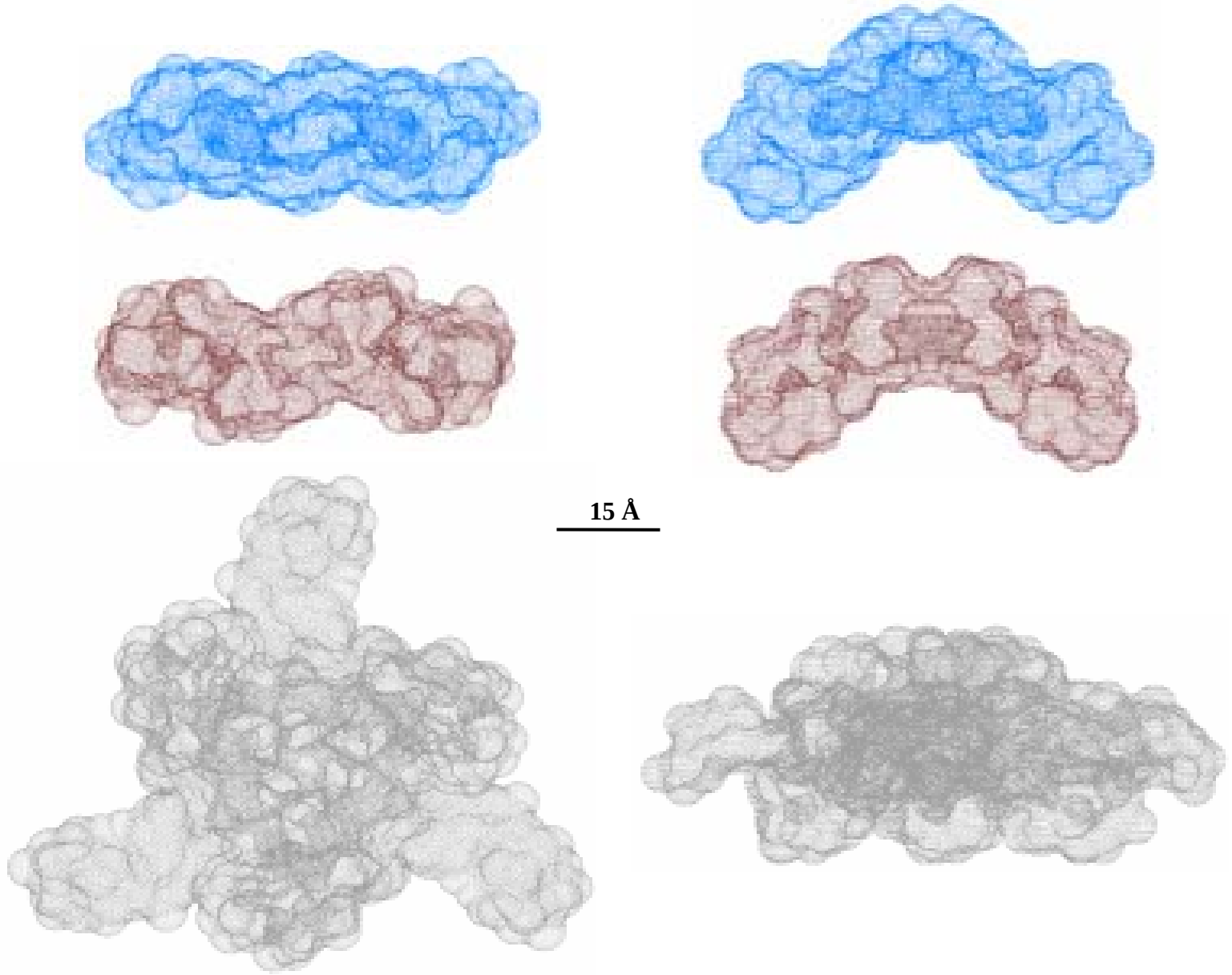
SAXS Analysis



Tim9

Tim10

Tim9-Tim10 complex



Acknowledgments

Collaborators

- **University of Manchester**

Present: Stephanie Agius
Felicity Alcock
Scott Allen
Hui Lu
Maïlys Vergnolle

Past: Sabrina Dyall (UCLA, USA)
Pierre Luciano (CNRS,Marseilles France)
Peter Savory (Scotland Diagnostics)
Sarah Vial (Vertex Pharmaceuticals, Oxford UK)

IMBB-FORTH
Catherine Baud

- **Daresbury Laboratory**

Günter Grossmann

- **UMIST**

Alexander Golovanov, Lu-Yun Lian

Funding

The Lister Institute, The Welcome Trust, MRC, BBSRC, EU, The Leverhulme Trust, The Royal Society