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UniMI participates in «Science without frontiers» program:
exchange program with Brasil (CAPES).
http://www.cienciasemfronteiras.gov.br/web/csf-eng/

My Research Interests:

- enzyme mechanisms

- regulation of activity

Main projects

- Structure-function relationships of flavin- and iron-sulphur-dependent oxidoreductases.

Other/Collaborative projects

- *Mycobacterium tuberculosis* enzymes acting on tRNA's as potential drug targets: Glu-tRNA synthetase & Glu-tRNA reductase.

- Folding Inhibitors of HIV-1 protease

Our approach:

gene cloning, engineering & expression

- protein (over)production & purification
- structure-function studies

– steady-state & pre-steady-state kinetics

– mechanistic studies

– absorbance & fluorescence spectroscopies

– Through collaborations:

– EPR, NMR, X-ray crystallography, Small-angle X-ray scattering, Cryoelectron microscopy , Molecular dynamics

Sample preparation and characterization

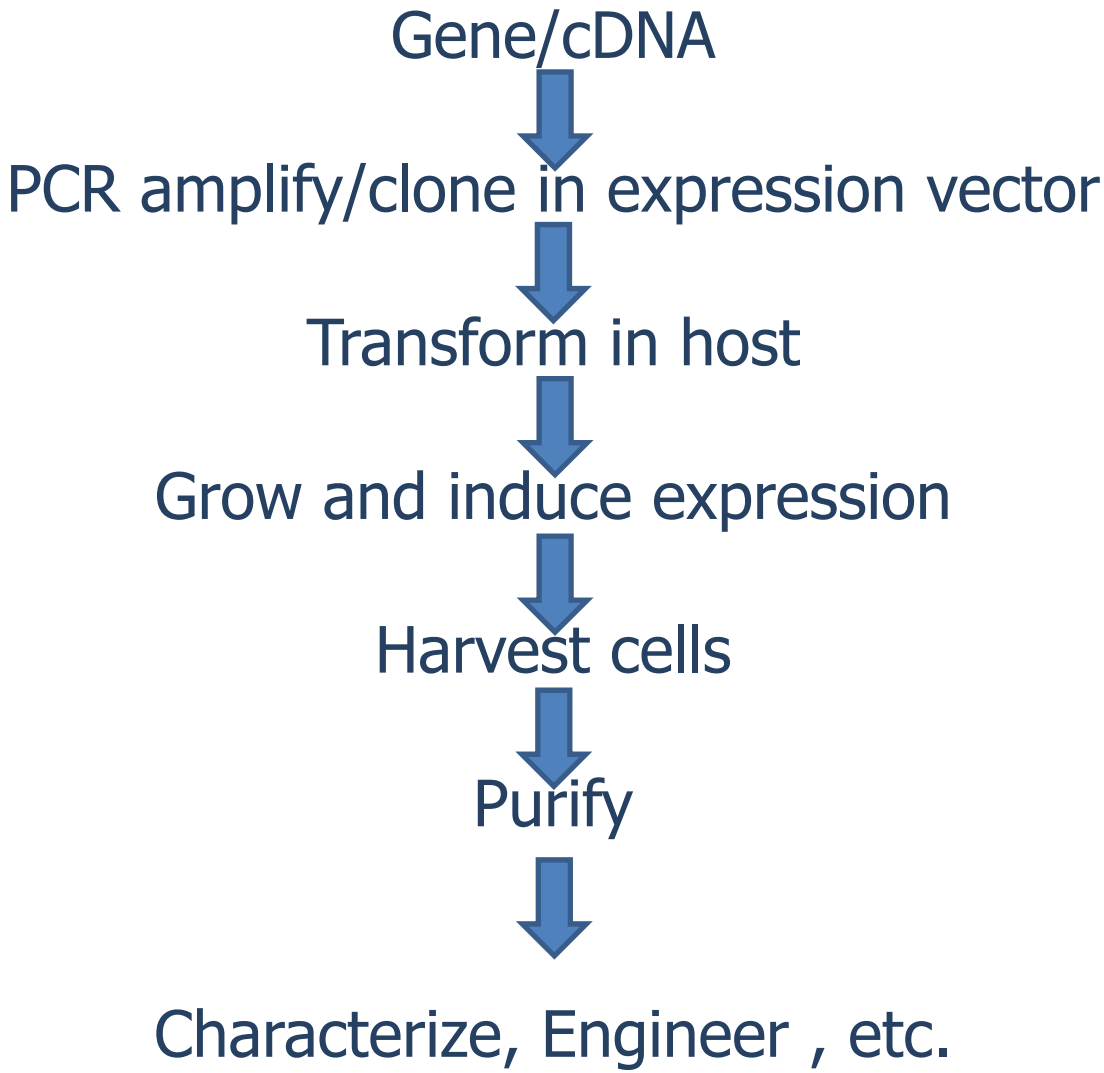
Structure-function studies require stable, reproducible, biologically active and homogeneous protein preparations.

Critical steps:

- Expression
- Purification
- Storage
- Protein concentration determination
- Cofactor/coenzyme content
- Chemical modifications
- Aggregation state
- Conformational changes
- Protein ligands
- Biological activity determination

An overview, some useful (?) references with examples (and troubleshooting)

Typical scheme for protein production in heterologous host



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Protein overproduction in heterologous host

Advantages:

- Cheap(er) and easy(ier) to handle starting tissue
  (e.g.: proteins from pathogens; proteins from human tissues)
- Large amounts of protein/g starting material
  (e.g.: 10 mg MTHFR from 10 kg pig liver)
- Protein engineering to facilitate purification, increase yield, limit protein degradation and/or denaturation to allow structure/ function studies.

Disadvantages:

- Post-translational modifications may not be reproduced
- Cofactor/coenzyme requirement may not be met by host
- Natural protein is part of a complex

Problems

- Protein is toxic to host
- Protein is insoluble

Protein overproduction in heterologous host - Troubleshooting

- Obtain information on natural protein and its protein/ligand partners directly (proteomic approaches) or by similarity (bioinformatics).

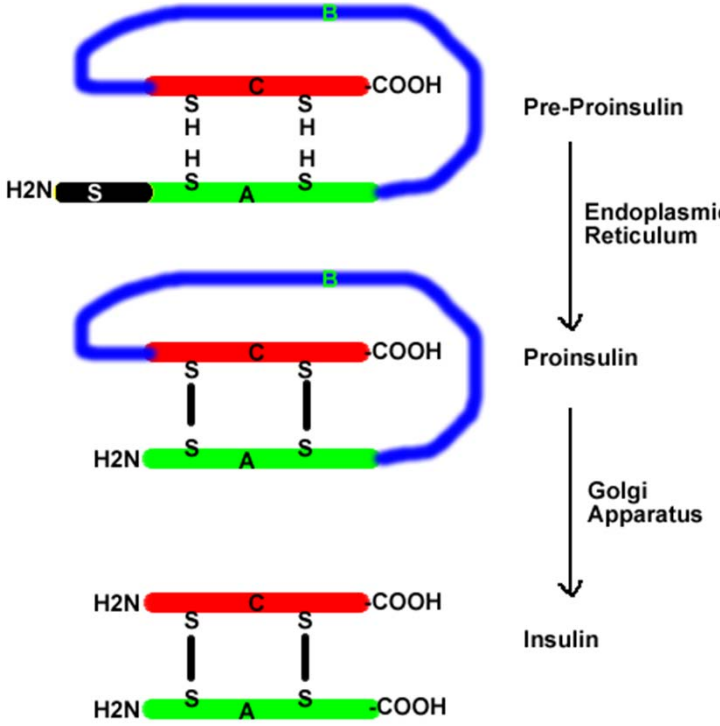
- Selection of the host (not only *E. coli*)
- Selection of the vector (not only pET vectors)
- DNA engineering (codon optimization)
- Protein engineering (addition of tags; removal of membrane-anchoring regions)
- Selection of the inoculum (e.g.: high starting cell concentration for toxic proteins) culture (minimal vs complete medium; low temperature), induction (IPTG, arabinose, T shift...) conditions

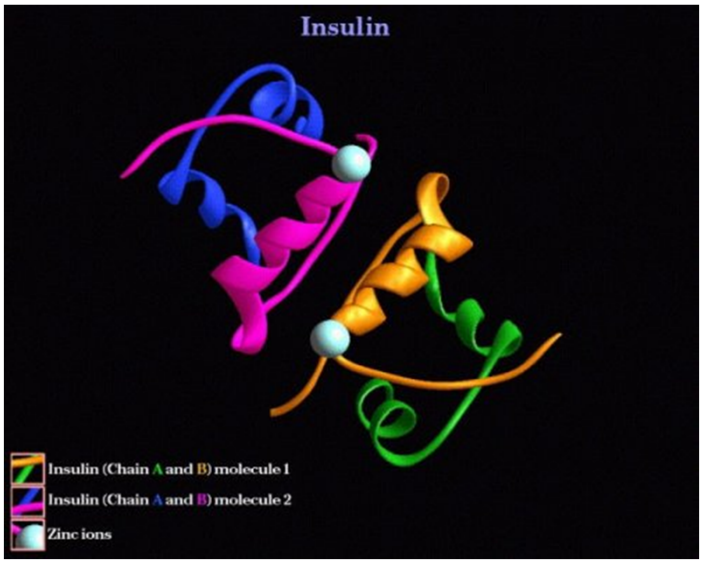
- Coexpression with chaperon proteins (not only GroE), cofactor/coenzyme synthetizing enzymes, partner protein.
- Host metabolic engineering

A few examples

- Post-translational modifications may not be reproduced without the precise knowledge of the mature protein

Insulin





Examples

Cofactor/coenzyme requirement not met by host

Optimization of expression of human sulfite oxidase and its molibdenum domain in E. coli required inactivation of mobA, the gene encoding the enzyme that converts the Molibdo-pterin coenzyme of Sulfite oxidase into Molibdo guanidine dinucleotide.

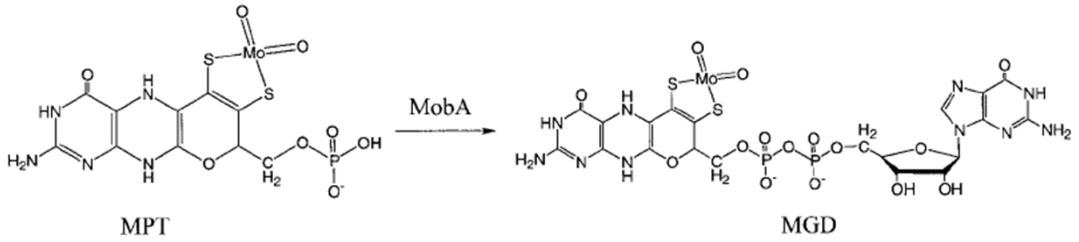


FIG. 1. The conversion of MPT to MGD in *E. coli* requires the product of the *mob* locus.

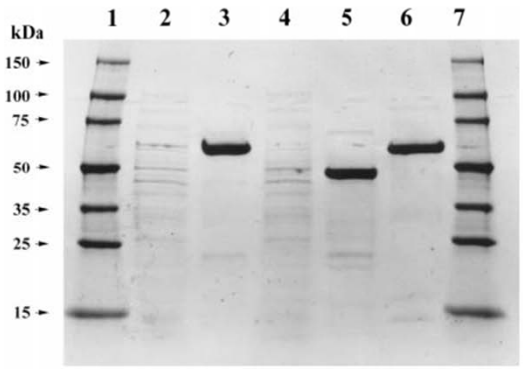
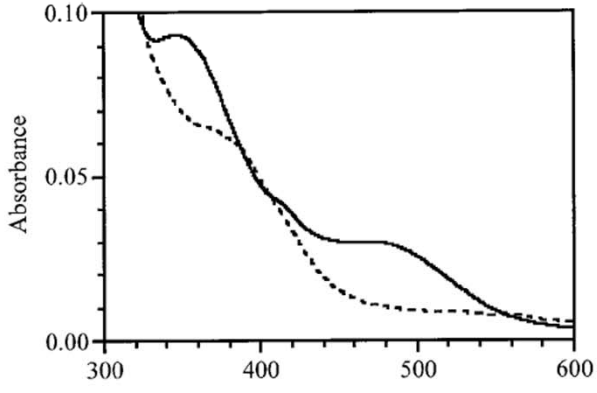


FIG. 2. SDS–polyacrylamide gel electrophoresis of purification of



Temple CA, Graf TN, Rajagopalan KV (2000) Optimization of expression of human sulfite oxidase. Arch. Biochem. Biophys. 383, 281-287.

Example: Natural protein is part of a complex

Expression in Pichia of zDHCR24 (the last enzyme of cholesterol biosynthesis) seems to be promoted by co-expression of DHCR7 (the enzyme catalyzing the previous step)

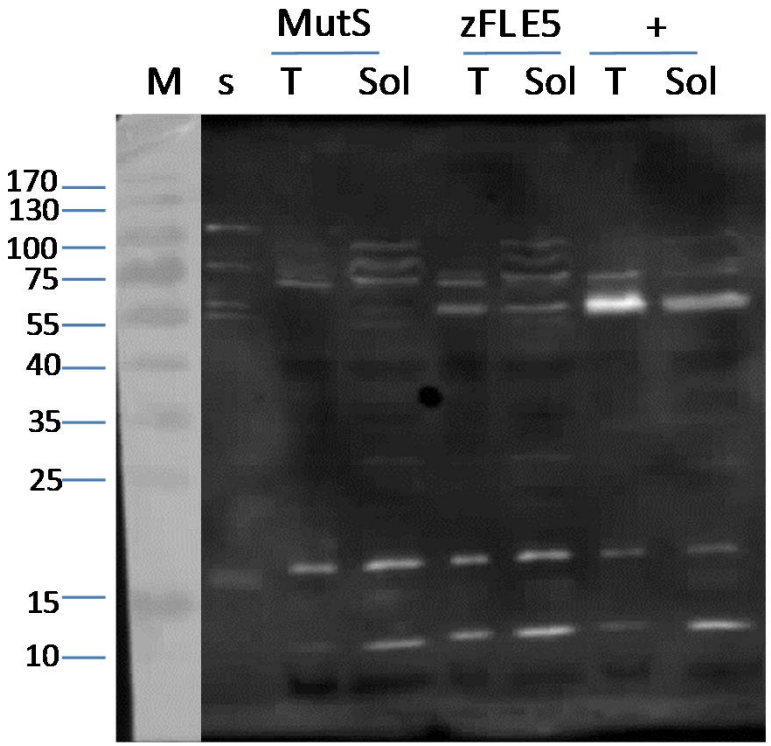


Figure 4: Levels of zDHCR24-His (clone E5) expressed in MutS strain in 50 ml of complete medium under AOX inducible promoter. Cells were disrupted under denaturing conditions to obtain the total extract (T) and under native conditions to obtain the soluble fraction by centrifugation at 13000 rpm, 15 min (Sol). M: molecular marker, s: standard mix of DHCR24 forms expressed in E. coli as inclusion bodies and resuspended in SB1X; -: MutS strain; +: MutS Δergs::DHCR7 Δerg6::DHCR24 strain grown in the same conditions.

Daniela Zucchini (MI), Andrea Camattari & Harald Pichler (Institute of Molecular Biotechnology, Graz, Austria)

Potential problems in Protein overproduction in E. coli that might be solved by switching to eukaryotic host coupled to metabolic engineering

Production of insoluble protein in E. coli may be due to :

- Too fast protein synthesis
- Too strong promoters

- No glycosylation, phosphorylation

- Lipid, sterol composition (especially for membrane proteins)

Available online at www.sciencedirect.com

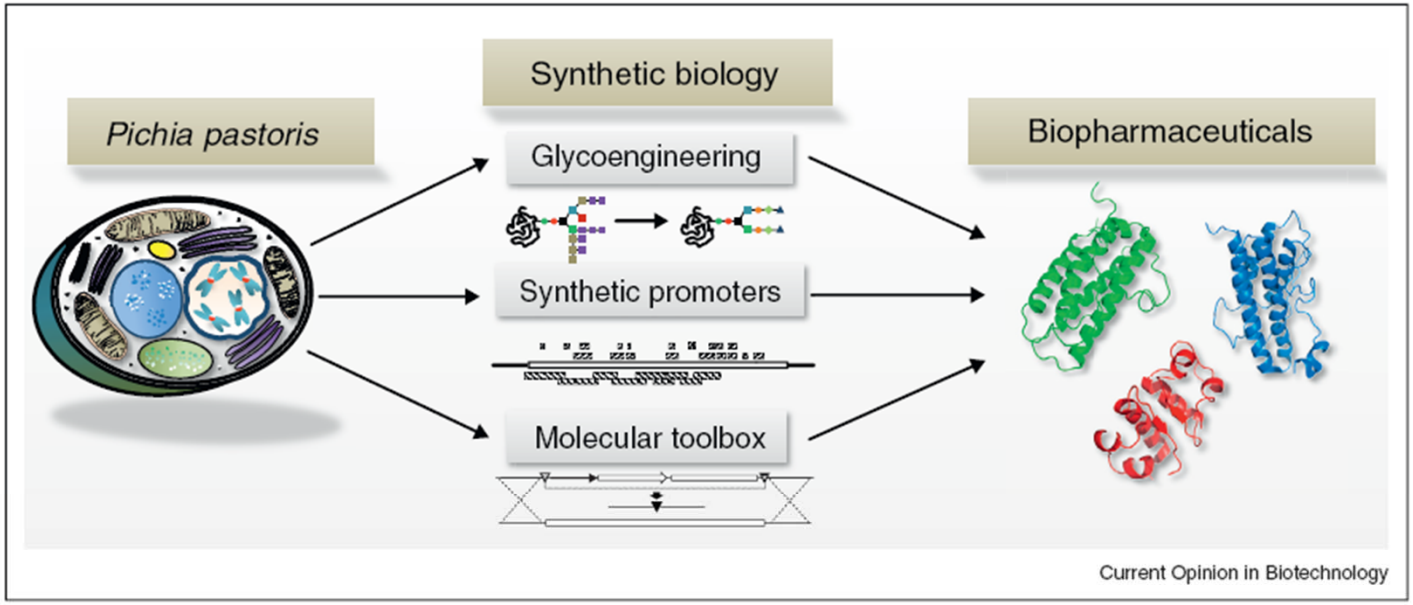
SciVerse ScienceDirect

Current Opinion in Biotechnology

New opportunities by synthetic biology for biopharmaceutical production in *Pichia pastoris*

Thomas Vogl[1], Franz S Hartner[2] and Anton Glieder[3]

Figure 1



Current synthetic biology approaches to improve biopharmaceutical yields and quality in *P. pastoris*. Glycoengineered strains provide humanized *N*-glycosylation patterns [14,15,16•], synthetic promoters allow the fine-tuning of expression levels [41,42,43•] and various tools for strain engineering [47–49,50•] and metabolic modeling [55•,56•,57•] are available.

Producing membrane proteins is still a major challenge due to the complexity of Folding/Trafficking/ maturation of membrane proteins and the requirement of many players

Review

Open Access

Tuning microbial hosts for membrane protein production

Maria Freigassner[1], Harald Pichler[1,2] and Anton Glieder*[1,2]

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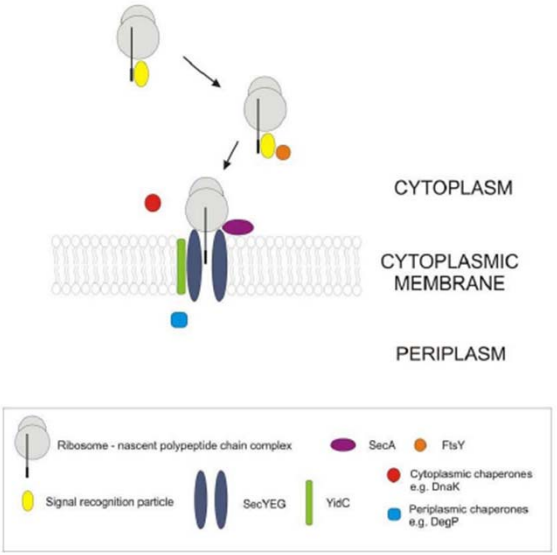

Figure 1
Membrane protein biogenesis in prokaryotes. In prokaryotes, most membrane proteins are targeted to and inserted into the cytoplasmic membrane by the SRP pathway, which involves interaction of the growing polypeptide chain with the signal recognition particle (SRP) and its receptor FtsY, binding of the nascent polypeptide chain-ribosome-complex to the SecYEG/YidC pore, translocation of cytoplasmic and periplasmic loops across the cytoplasmic membrane and their folding by SecA and various chaperones and insertion of hydrophobic segments into the membrane. The autonomous, YidC and Tat pathways, that are used by small proteins and membrane-associated periplasmic proteins, respectively, are mentioned here for sake of completeness.

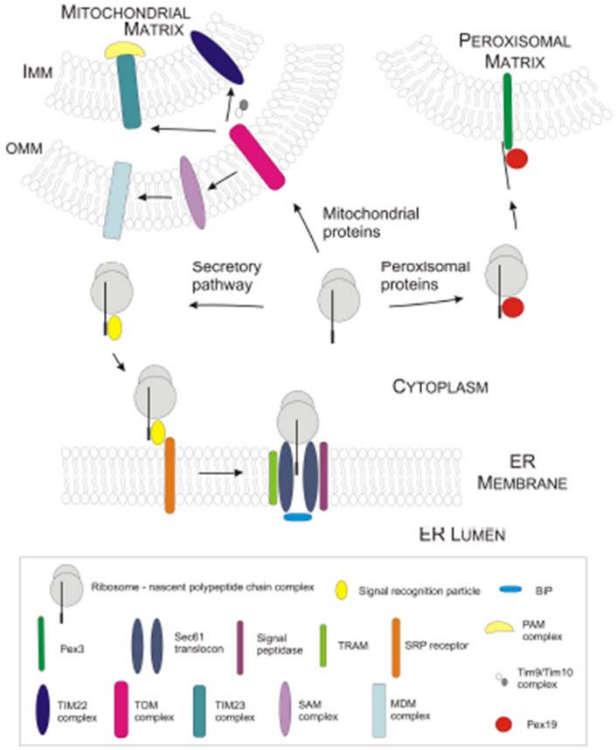

Figure 2 (see legend on next page)

Host Membrane composition may be critical for expression of mammalian membrane proteins

Appl Microbiol Biotechnol (2013) 97:9465–9478
DOI 10.1007/s00253-013-5156-7

APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

A novel cholesterol-producing *Pichia pastoris* strain is an ideal host for functional expression of human Na,K-ATPase α3β1 isoform

Melanie Hirz · Gerald Richter · Erich Leitner · Tamara Wriessnegger · Harald Pichler

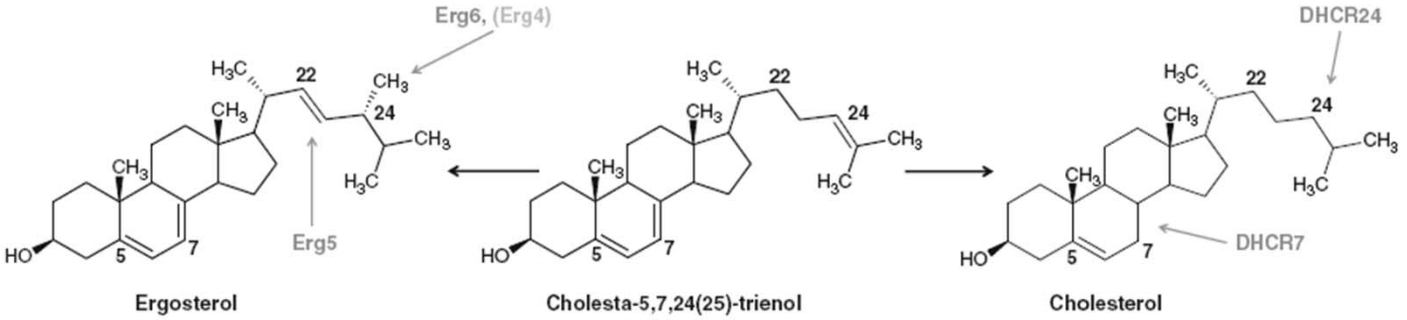

Fig. 1 Structures of ergosterol and cholesterol. The major yeast sterol, ergosterol, differs from the mammalian cholesterol lacking two double bonds at positions C-7 and C-22 and one methyl group at position C-24. The enzymes involved in ergosterol synthesis are the sterol C-22 desaturase encoded by *ERG5* and the sterol C-24 methyl transferase encoded by *ERG6*. For cholesterol synthesis, two dehydrocholesterol reductases, *DHCR7* and *DHCR24*, are required to saturate specifically the double bonds at positions C-7 and C-24. Cholesta-5,7,24(25)-trienol is shown as a theoretical, common biosynthetic intermediate of ergosterol and cholesterol biosynthesis. However, cholesta-5,7,24(25)-trienol is hardly detectable in ergosterol-producing yeast strains due to Erg6p action

Protein overproduction in heterologous host: Troubleshooting

Use different host/vector/construct combinations

High-throughput approach
+
Screening method for activity
And/or
Good antibodies for dot-blot (faster but less informative than western blot)

Try to produce same protein from different species

Go to specialised centers

Protein overproduction in heterologous host

Some (more) web sites/centers

EMBL Protein production facilities:
http://www.embl.de/pepcore/pepcore_services/
http://www.embl-hamburg.de/facilities/spc/index.html
http://www.embl.fr/services/ht_expression/index.html

Oxford Protein Production facility:
http://www.oppf.rc-harwell.ac.uk/OPPF/

Instruct Centers:
http://www.structuralbiology.eu/resources/instruct-centres

Wolfson Centre for Applied Structural Biology , Hebrew University of Jerusalem:
http://wolfson.huji.ac.il

Membrane Protein Expression Center (MPEC) UCSF :
http://mpec.ucsf.edu/index.htm

ACIB/TU Graz : Institute of Molecular Biotechnology:
http://www.imbt.tugraz.at/

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Purification (not only IMAC)

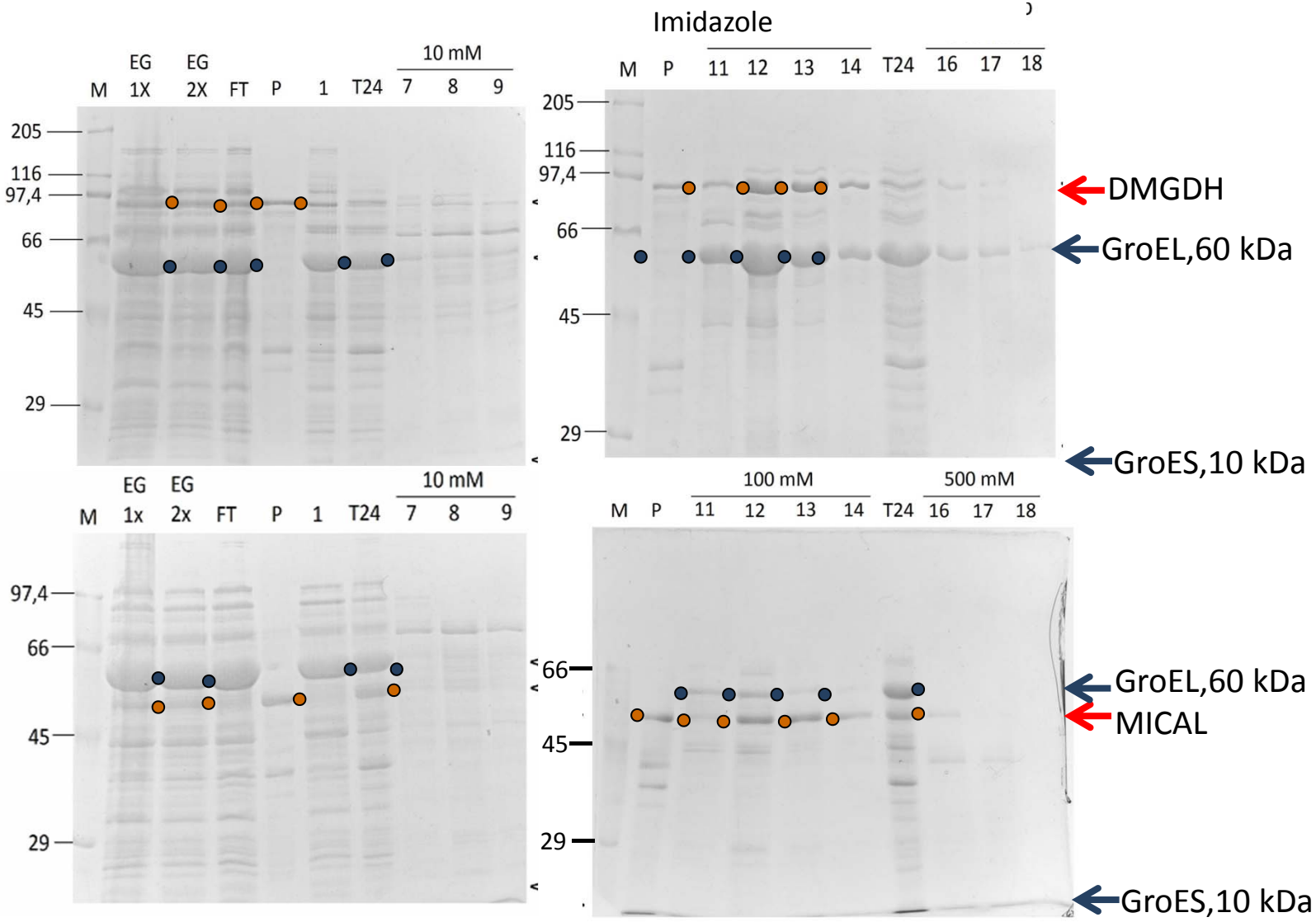
- Ni-NTA-Sepharose:
- Overload the column
- Keep pH 7.5 – 8.0
- Keep low concentration of reducing agent (try to avoid protein oxidation from Ni^{+}/Ni^{++} ions leaching from resin)

- Several E. coli proteins will bind to Ni-resin
- Chaperons used to promote expression may co-elute (bad sign; see DMGDH vs MICAL)

- Gel filtration

- Ion exchange chromatography

Ni-NTA Sepharose of protein coproduced with GroE (or other chaperons) Coproduction of human dimethylglycine dehydrogenase or MICAL with GroE in E. coli BL21(DE3) and Ni affinity chromatography: coelution with GroE only with DMGDH suggests some misfolded protein?



Gel filtration reveals non specific aggregates or multiple oligomerization states

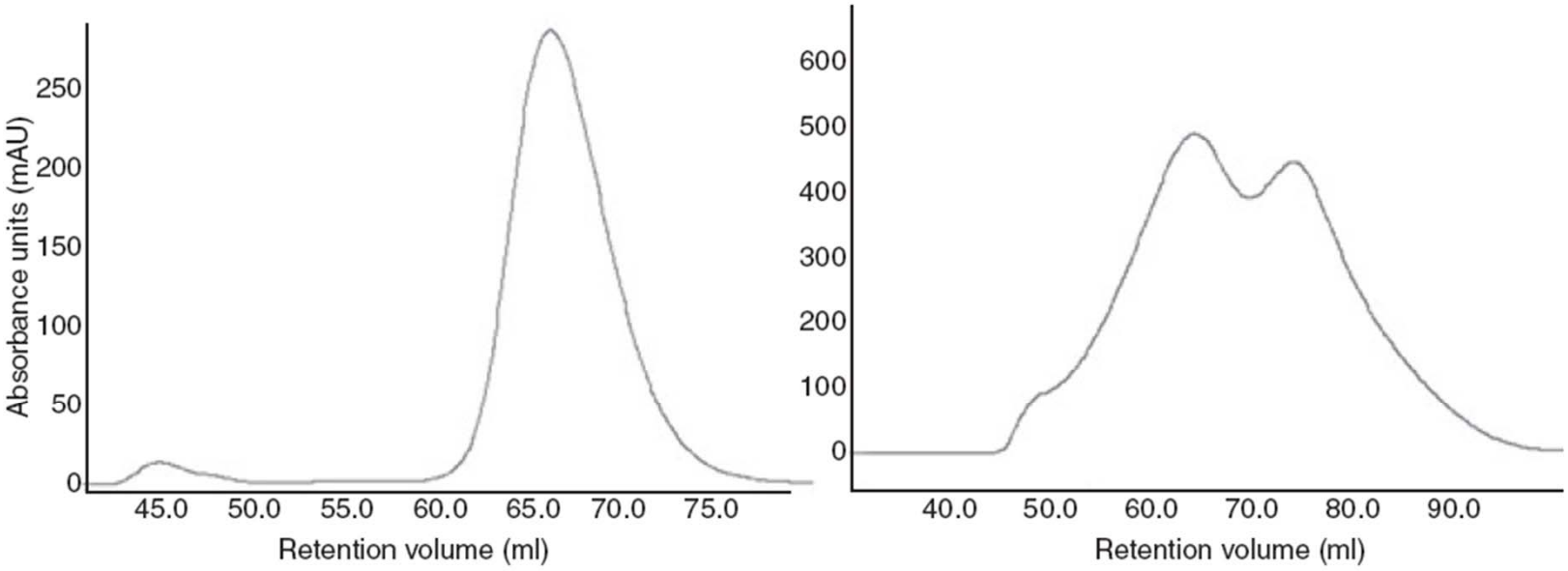
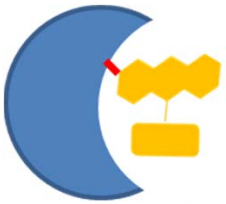


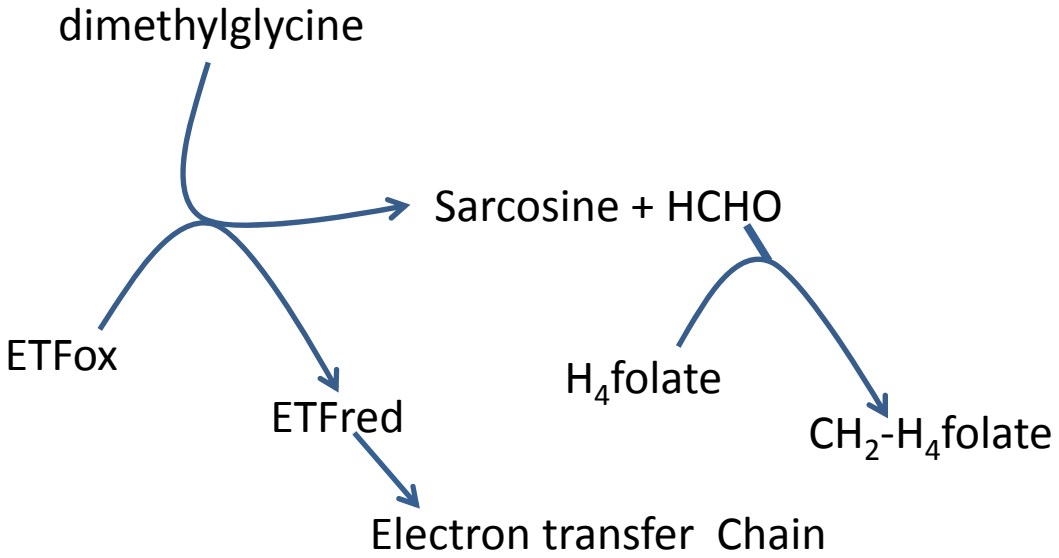
Figure 2 | Gel filtration profiles. Representative good (left) and bad (right) gel-filtration profiles of two different proteins purified on an ÄKTAxpress system using a HiLoad Superdex 200 column (GE Healthcare).

Nature methods (2008) 5, 135

Ion exchange chromatography revealed multiple forms of hDMGDH (dimethylglycine dehydrogenase), which otherwise (gel filtration, affinity) seemed homogeneous



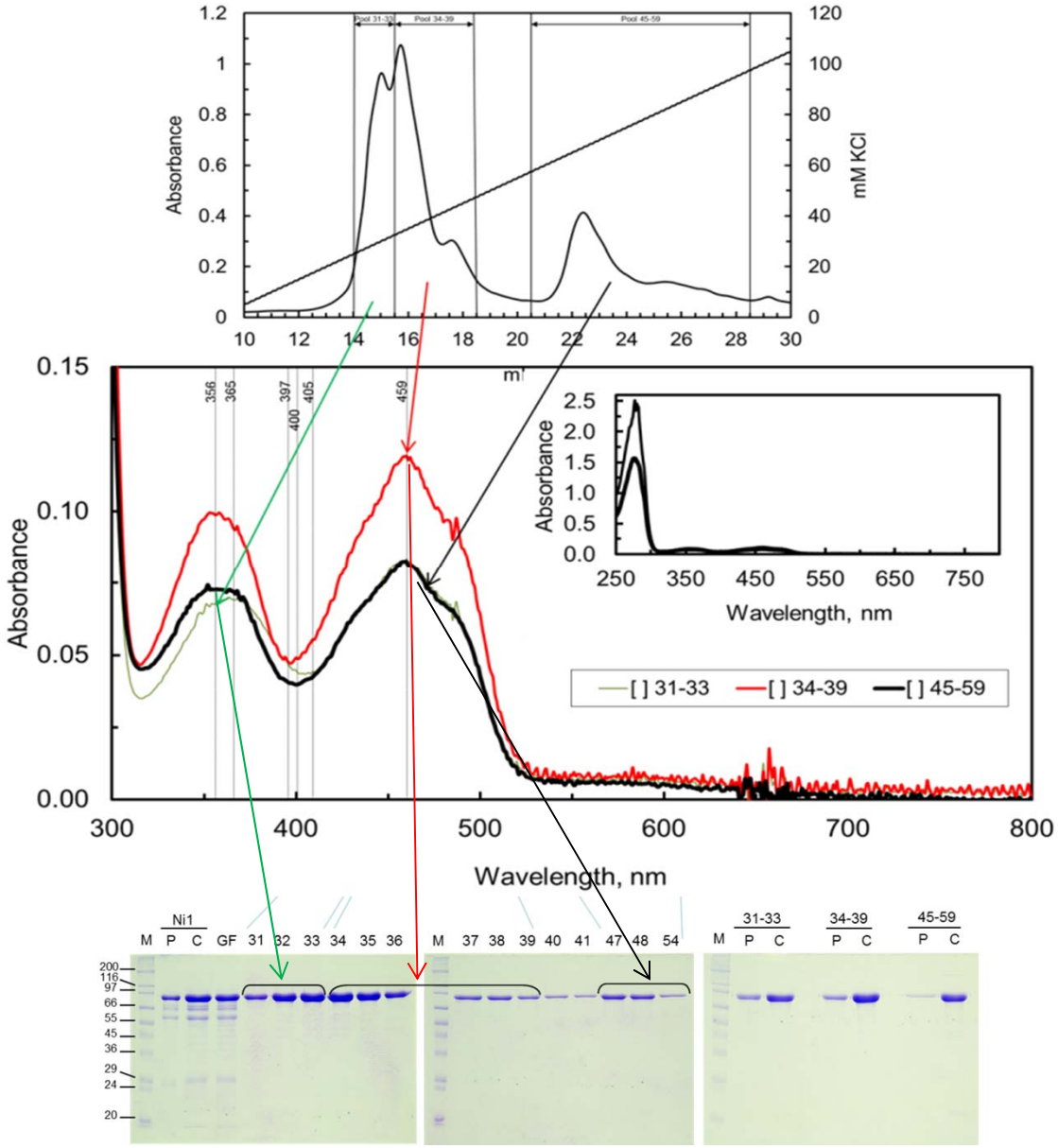
Holo-DMGDH
(covalently bound FAD)



Dimethylglycine dehydrogenase: FAD dependent dehydrogenase containing covalently bound FAD. Same covalent bond found in succinate dehydrogenase, fumarate reductase. DMGDH feeds 1 carbon unit into folate-dependent cycle; defects of DMGDH: diseases belonging to broad class of «mitochondrial diseases»

MonoQ chromatography of hDMGDH from IMAC:

multiple forms of hDMGDH, which can be distinguished on the basis of the absorbance spectra in the visible region thanks to the sensitivity of the FAD spectrum to the environment.



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Sample storage
Suggestions from A. Fersht laboratory:
Flash freeze in liquid N_2 and store at -70°C - -80 °C (also to block proteases) or at -20°C in 50 % glycerol

Freezing and Storing Protein Solutions

The following quote from the practical notes given to all members of Professor Sir Alan Fersht's research group gives some very important and useful information on the freezing and storage of protein solutions. Failure to heed this advice can result in change or loss of activity, unfolding, and/or aggregation of stored protein solutions.

Never freeze and thaw solutions of proteins at $-20°C$. Flash freeze in small aliquots in liquid N_2 and then store at $-70°C$ or below, never at $-20°C$. This is because there is a phase change in ice at $-70°C$ to $-80°C$. The higher temperature form is unkind to proteins. Take care to thaw protein solutions slowly. Always check by repeated cycles of thawing and freezing that the protein is stable. Sometimes the addition of 5% glycerol or sucrose or various salts is sufficient to stabilize a protein to freezing in liquid N_2. An alternative procedure is to store in 50% glycerol/water at $-20°C$, above the freezing point of the mixture.

Sample storage: keep your protein concentrated (to avoid loss through adsorption to vessel) but not too much (to avoid aggregation)

The practical notes also offer the following advice on the use and storage of dilute protein solutions. Loss of material due to surface adsorption is a frequent cause of aberrant stoichiometry in titrations, and these techniques can help to ameliorate this effect.

> *Many enzymes that are stable at high concentrations appear to be unstable when used or stored after dilution. This is often because of adsorption to the sides of vessels (tubes, microplates, pipette-tips). The following procedures help:*
>
> 1. *Siliconize all vessels used.*
> 2. *Add BSA to 50 µg/mL to buffer.*
> 3. *Add Tween 20 or 80 to 0.05%.*

Not for structural work!

If you are worried about the long-term stability of a protein solution, or observe a lack of reproducibility in repeated assays from frozen stock solutions, there are a number of methods that you can use to test whether your protein is robust with respect to freezing, storage and thawing:

Sample storage: check stability through activity measurements, SDS-PAGE, but also absorbance spectra and dynamic light scattering before and after (ultra)centrifugation

1. Perform an activity or binding assay using a standard stock of a stable substrate or binding partner. Check that all of the parameters of the assay (start and end signals, dissociation or kinetic constants) are reproducible. Large changes in any parameter may indicate a change in the condition of the protein caused by freezing, storage or thawing.

2. Measure the protein concentration by spectrophotometry before and after freezing, storage and thawing. Pay particular attention to the apparent absorbance signal at 330 nm, which arises from scattering by aggregates. Read the protocol on measuring protein concentration for more information about this.

3. Use dynamic light scattering (DLS) to assess the particle size of the solution before and after freezing, storage and thawing. This technique is very sensitive to the presence of aggregates and is very useful for monitoring changes to stock solutions over time.

Sample shipping in dry ice: avoid sample acidification!

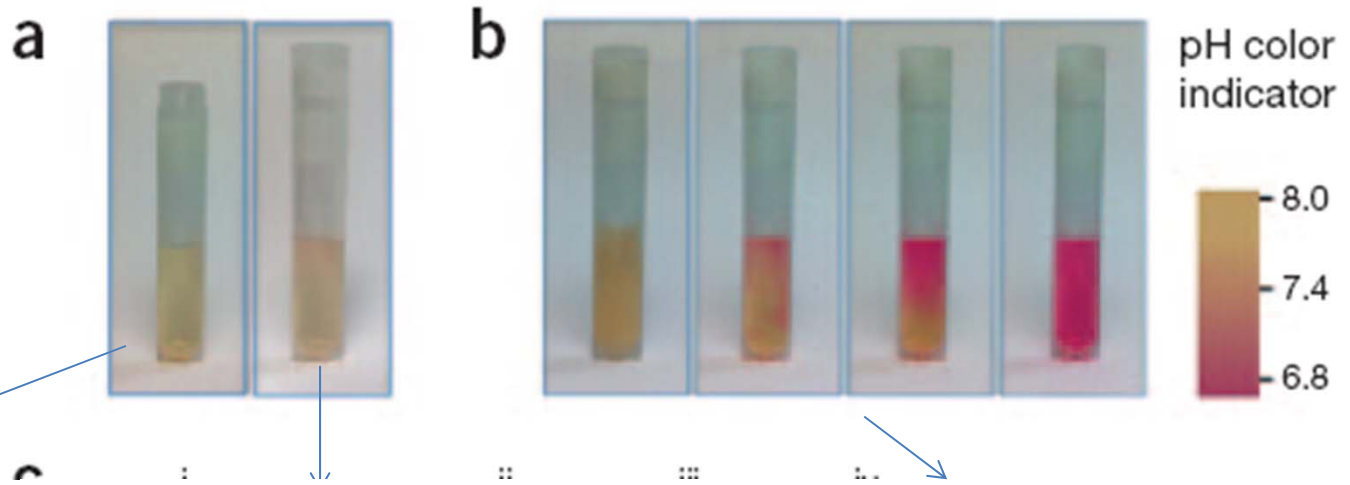
Dry ice = CO_2 $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$

Murphy et al. (2013) Protein instability following transport or storage in dry ice. Nature methods, 10,

Tests:

Freezing & storage: 48 h @ -70°C + 48 h in dry ice

Thawing conditions:



Remove cap before thawing

Store @ -70°C for 96 h before thawing

Otherwise: acidification

- Test your container;

- Sealing with Parafilm does not seem to work

- Ziplock mylar bag does not seem to work

Parafilm + Heat-seal in hybridization bag + vent your thermos during long trip (+ overnight storage at -80°C) seems to work (for MAV)

Alt/ seal dry ice in bags (this is how dry ice is carried in airplanes to keep food frozen!) but avoid explosions

Supplementary Table 1: Integrity Testing of Container/Closure Systems

Container-Closure System		Materials	Closure	pH after storage*	
Container type	Brand / Part No.	Body/Cap/Seal	type/geometry	-70°C	Dry Ice
Cryogenic storage vials (4.5 - 5.0) mL	Nunc / 337516	PP / PE / **	threads/external	> 8.0 > 8.0	< 6.9 ~ 7.7
	Nunc / 379146	PP / PE / SL	threads/internal	> 8.0 > 8.0	< 6.9 < 6.9
	Corning / 430663	PP / PP / SL	threads/external	> 8.0 > 8.0	< 6.9 < 6.9
	Corning / 430656	PP / PP / SL	threads/internal	> 8.0 > 8.0	< 6.9 < 6.9
Conical bottom centrifuge tubes (15 mL)	Nunc / 339650	PP / PE / **	threads/external	> 8.0 > 8.0	< 6.9 < 6.9
	VWR / 89039-666	PP / PP / **	threads/external	> 8.0 > 8.0	< 6.9 < 6.9
	Corning / 430055	PT / PE / **	threads/external	> 8.0 > 8.0	< 6.9 > 8.0
Micro tubes (1.5 mL)	Eppendorf / 22-36-41-11	PP / PP / **	snap/internal	> 8.0 > 8.0	~ 7.5 < 6.9
Glass vials (7 - 8) mL	Supelco / 27150-U	BG / MF / TF	threads/external	> 8.0 > 8.0	< 6.9 < 6.9
	Wheaton/ W224584	BG / PH / SB	threads/external	> 8.0 > 8.0	> 8.0 < 6.9

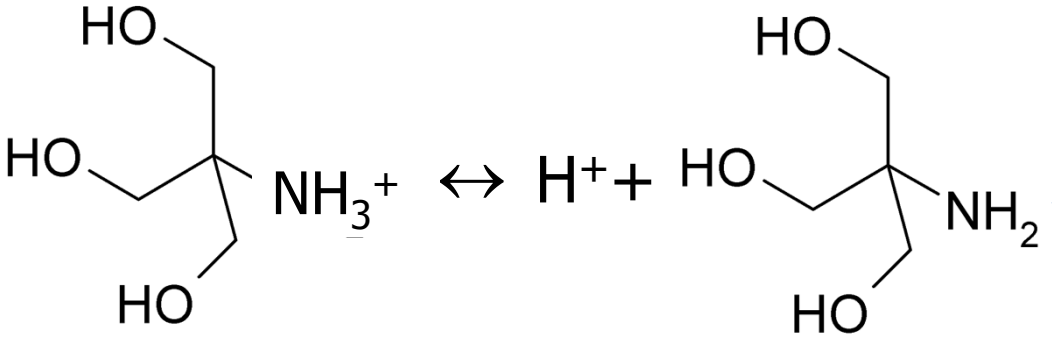
Notes and abbreviations:

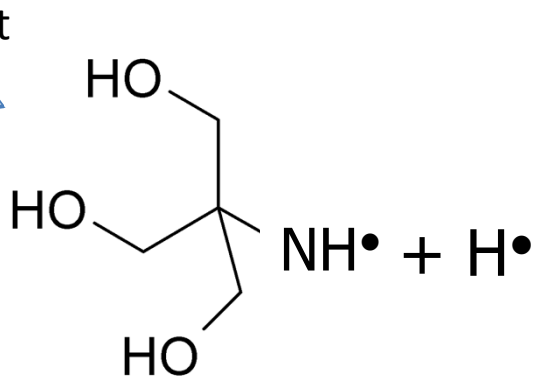
* = pH determined via buffered colorimetric indicator solution placed in each vessel.
** = Seal is accomplished through an press fit between the cap and body.

BG = borosilicate glass
MF = melamine formaldehyde
PE = polyethylene (high density)
PF = phenol formaldehyde
PP = polypropylene
PT = polyethylene terephthalate
SB = styrene-butadiene rubber
SL = silicone rubber
TF = polytetrafluoroethylene

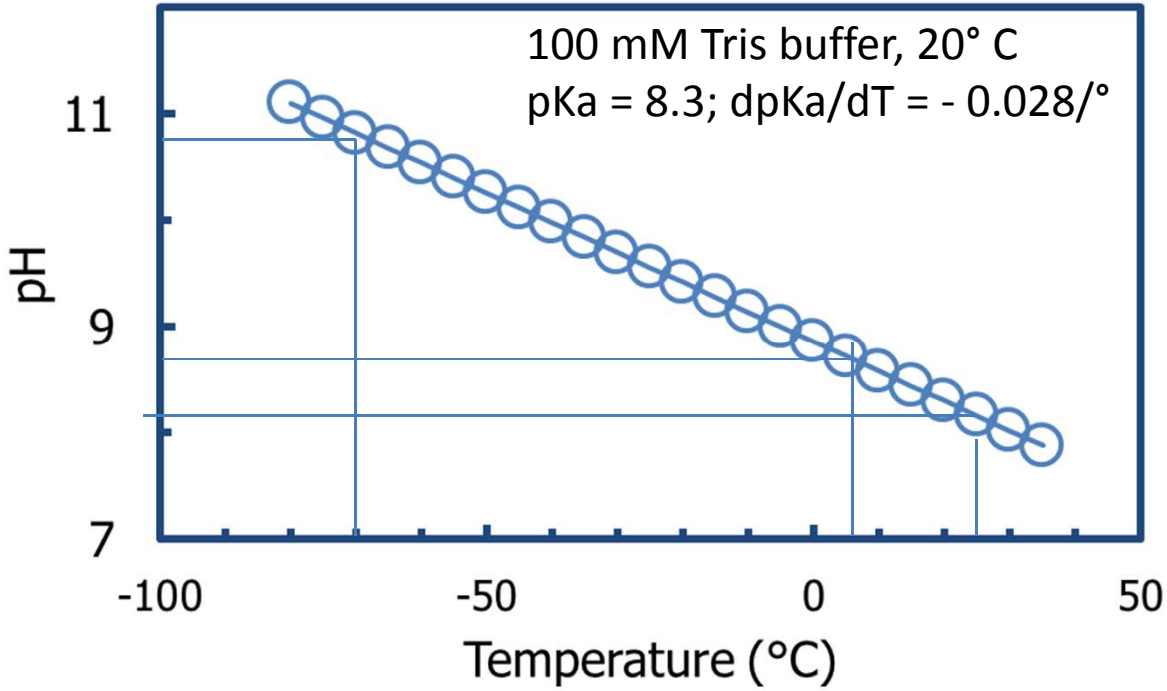
Sample storage:

Tris buffers are very common, but be careful to pH shift and photochemical reactions!





pKa of Tris is temperature sensitive



Tris, as an amine, reacts with light and forms radicals (e.g. Flavin photoreduction)

Protein concentration determination:
critical for stoichiometry (cofactor content, ligand binding), specific activity, mass/shape determination by SAXS

UV absorbance (computed/determined*)
Vis absorbance (if chromophore is present and extinction coefficient is known*)

*Colorimetric method:

- Biuret:
- Lowry
- Bradford
- BCA
- 660 dye

Protein concentration determination: theoretical for protein without chromophor/ligand

http://web.expasy.org/protparam/

The principle:

$$\varepsilon_{280} = n_{Tyr} * \varepsilon_{280,Tyr} + n_{Trp} * \varepsilon_{280,Trp} + n_{cystine} * \varepsilon_{280,\ cystine}$$

Where:

$\varepsilon_{280,Tyr} = 1490\ M^{-1}cm^{-1}$, $\varepsilon_{280,Trp} = 5500\ M^{-1}cm^{-1}$ $\varepsilon_{280,cystine} = 125\ M^{-1}cm^{-1}$

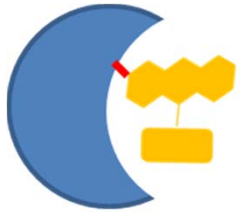
With: $\varepsilon_{280,Tyr}$ and $\varepsilon_{280,Trp}$ calculated at pH 6.5, in 6.0 M guanidium hydrochloride, 0.02 M phosphate buffer.

Two values: one assuming that all Cys are free, one assuming that all Cys form SS bonds

Gu/HCl should have little effect on ε_{280} , but better check with denatured and dialysed protein

Protein concentration determination: theoretical for protein with chromophor –

Example: DMGDH, an enzyme containing covalently bound FAD



$$\varepsilon_{280} = n_{Tyr}{*}\varepsilon_{280,Tyr} + n_{Trp}{*}\varepsilon_{280,Trp} + n_{cystine}{*}\varepsilon_{280,\ cystine} + n_{FAD}{*}\varepsilon_{280,\ FAD}$$

$$n_{FAD} = 1 \text{ for holo-DMGDH}$$

- $\varepsilon_{280,\ FAD:}$ determine experimentally in buffer + GuHCl
-

http://web.expasy.org/protparam/ output

Number of amino acids: 861
Molecular weight: 96236.7
Theoretical pI: 6.74

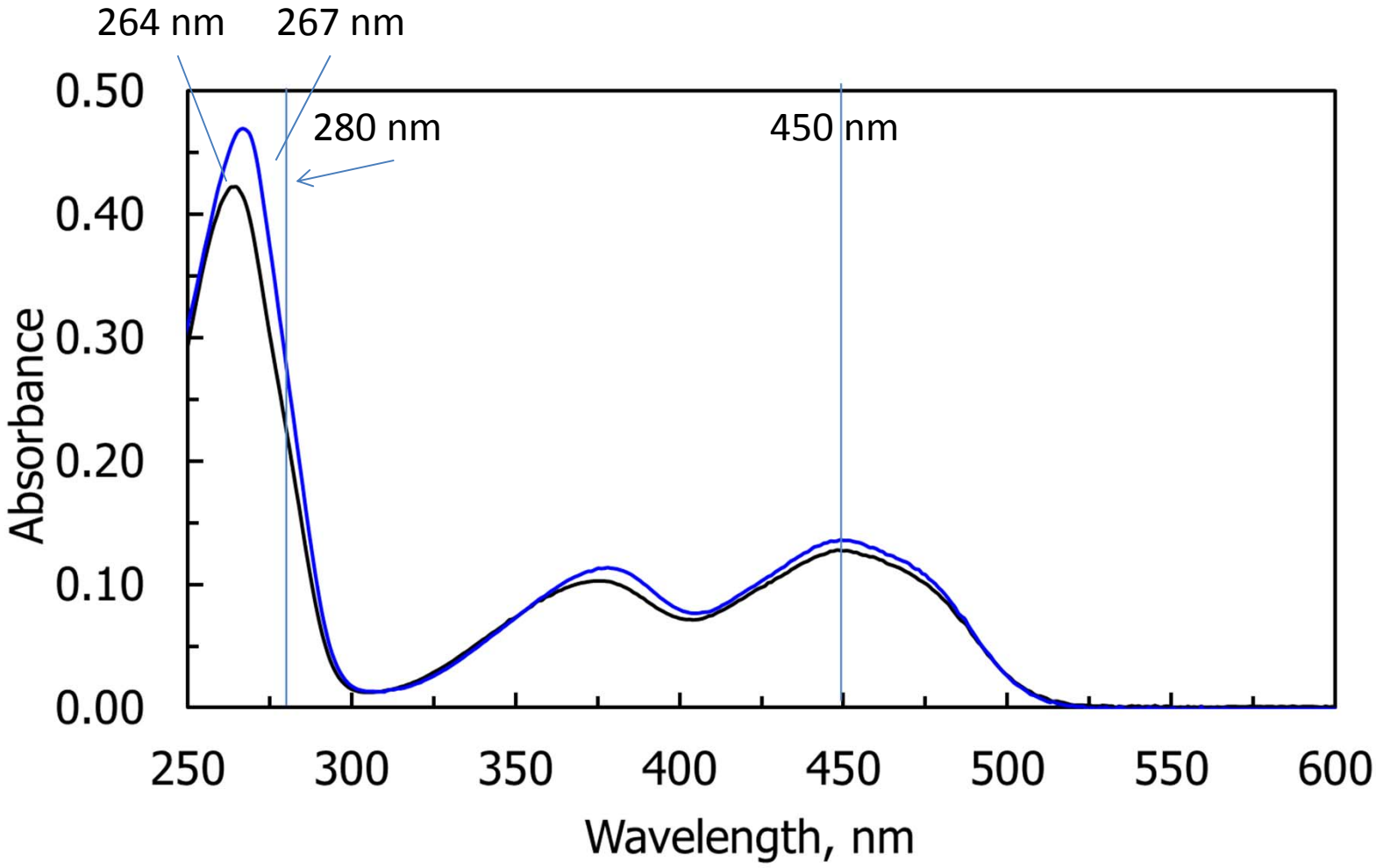
Extinction coefficients:

Extinction coefficients are in units of M^{-1} cm^{-1}, at 280 nm measured in water.

ε_{280} = 143505 M^{-1} cm^{-1} assuming all pairs of Cys residues form cystines (Abs 0.1% (=1 g/l) 1.491)

ε_{280} = 143130 M^{-1} cm^{-1} assuming all Cys residues are reduced (Abs 0.1% (=1 g/l) 1.487)

Determine $\varepsilon_{280,\ FAD}$ experimentally in buffer + GuHCl using known ε at 450 nm of FAD in buffer



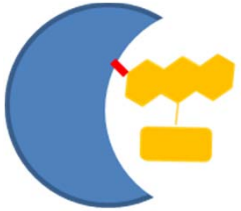
	buffer	Buffer + GuHCl
$\varepsilon_{448,\ FAD}$	11.3	11.9
$\varepsilon_{280,\ FAD}$	19.95	24.32

Calculation of protein extinction coefficient taking into account the bound cofactor/coenzyme: use extinction coefficients of protein (from Protparam) AND coenzyme in guanidine to minimize spectral perturbation due to protein environment; calculate/compare in GuHCl

Apo-DMGDH

DMGDH●FAD
(non covalent)

Holo-DMGDH
(covalently bound
FAD)



A280-protein	A280_FAD	A280- EFAD	UV/Vis	f(holo)
143.13	24.32	167.45	14.0714286	1
143.13	21.888	165.018	15.4078431	0.9
143.13	19.456	162.586	17.0783613	0.8
143.13	17.024	160.154	19.2261705	0.7
143.13	14.592	157.722	22.089916	0.6

Protein concentration determination – Common methods based on different principles

	Lower limit of calibration curve (µg in 1ml assay)	Sensitivity to Protein aa composition	Sensitivity	Detection of interference/Troubleshooting
Biuret	15	low	More or less all are sensitive to buffer, reducing agents, detergent, denaturants (guanidine)!	- Check extent of interference by e.g. Your solvent - Include your solvent atfixed concentration in references and samples.
Lowry	10	low		
Bradford	1	high		
BCA	1	low		
660 dye	1	low(?)		

*: commercial formulations; see also: http://wolfson.huji.ac.il

Quick Technical Summaries – Thermo Scientific Protein Assays

Working Range (sample volume)*	Characteristics/Advantages	Applications	Disadvantages	Interfering Substances
Pierce® 660nm Protein Assay				
Standard Protocol: *25-2,000µg/mL (65µL)* **Microplate Protocol:** *50-2,000µg/mL (10µL)*	Compatible with reducing agents, chelating agents and detergents Faster and easier to perform than BCA or Coomassie (Bradford) Assays Excellent linearity of color development within the detection range Less protein-to-protein variability than the Coomassie (Bradford) Assay Reaches a stable end point Compatible with Laemmli sample buffer containing bromophenol blue when using Compatibility Buffer	Ideal for measuring total protein concentration in samples containing both reducing agents and detergents Used for quick, yet accurate estimation of protein	Use reagent with IDCR (Ionic Detergent Compatibility Reagent) with samples containing ionic detergents like SDS Greater protein-to-protein variability than the BCA Assay	High levels of ionic detergents require the addition of the Ionic Detergent Compatibility Reagent (IDCR).
The BCA Protein Assay - Reducing Agent Compatible				
Standard Protocol: *125-2,000µg/mL (25µL)* **Microplate Protocol:** *125-2,000µg/mL (9µL)*	Compatible with up to 5mM DTT, 35mM 2-Mercaptoethanol or 10mM TCEP No protein precipitation involved Sample volume only 9µL (microplate protocol) Compatible with most detergents Significantly less (14-23%) protein:protein variation than Bradford-based methods	Allows the use of the superior BCA Assay in situations in which it is normally unable to be read No precipitation step means no worries about difficult-to-solubilize proteins	Requires heating for color development	Compatible with all reducing agents and detergents found at concentrations routinely used in protein sample buffers
The BCA Protein Assay				
Standard Protocol: *20-2,000µg/mL (50µL)* **Enhanced Standard Protocol:** *5-250µg/mL (50µL)* **Microplate Protocol:** *20-2,000µg/mL (25µL)*	Two stable reagents used to make one working reagent Working reagent stable for one week at room temperature Compatible with detergents Simple, easy to perform Less protein:protein variation than Coomassie dye methods Works with peptides (three amino acids or larger) Flexible incubation protocols allow customization of reagent sensitivity and working range	Adaptable for use with microplates Determine the amount of IgG coated on plates Measure the amount of protein covalently bound to affinity supports Determine copper levels using a reagent formulated with BCA Reagent A[4]	Not compatible with thiols/reducing agents Requires heating for color development Not a true end-point assay	Reducing sugars and reducing agents Thiols Copper chelating agents Ascorbic acid and uric acid Tyrosine, cysteine and tryptophan 50mM Imidazole, 0.1M Tris, 1.0M glycine
The Micro BCA Protein Assay				
Standard Protocol: *60°C for 60 minutes 0.5-20µg/mL (0.5mL)* **Microplate Protocol:** *37°C for 120 minutes 2-40µg/mL (150µL)*	Three stable reagents used to make one working reagent Working reagent stable for 24 hours at room temperature Compatible with most detergents Simple, easy to perform Less protein:protein variation than BCA, Coomassie dye or Lowry Methods Works with peptides (three amino acids or larger) Linear color response to increasing protein concentration	Suitable for determining protein concentration in very dilute aqueous solutions Adaptable for use with microplates[1]	More substances interfere at lower concentrations than with BCA Assay because the sample volume-to-reagent volume ration is 1:1 60°C water bath is needed	Reducing sugars and reducing agents Thiols Copper chelating agents Ascorbic acid and uric acid Tyrosine, cysteine and tryptophan 50mM Imidazole, 0.1M Tris, 1.0M glycine

** Sample volume per 1mL total assay volume for measurement in 1cm cuvette (Standard Protocol). Sample volume per 200-300µL total volume for measurement in 96-well microplate.*

Protein concentration determination – Troubleshooting

	Lower limit of calibration curve (µg in 1ml assay)	Sensitivity to Protein aa compositi on	Sensitivity	Detection of interference/Troubleshooting
Biuret	15	low	More or less all are sensitive to buffer, reducing agents, detergent, denaturants (guanidine)!	- Use 3-5 different protein quantities and check linearity. Intercept should be zero. - Check effect of your buffer added in a fixed amount in Std curve and your samples - Pre-precipitate protein* (make sure it is re-solubilized prior to assay) - Does your protein precipitate in assay? (check effect of order of reagents addition)
Lowry	10	low		
Bradford	1	high		
BCA	1	low		
660 dye	1	low(?)		

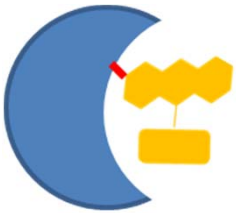
*: commercial formulations; see also: http://wolfson.huji.ac.il

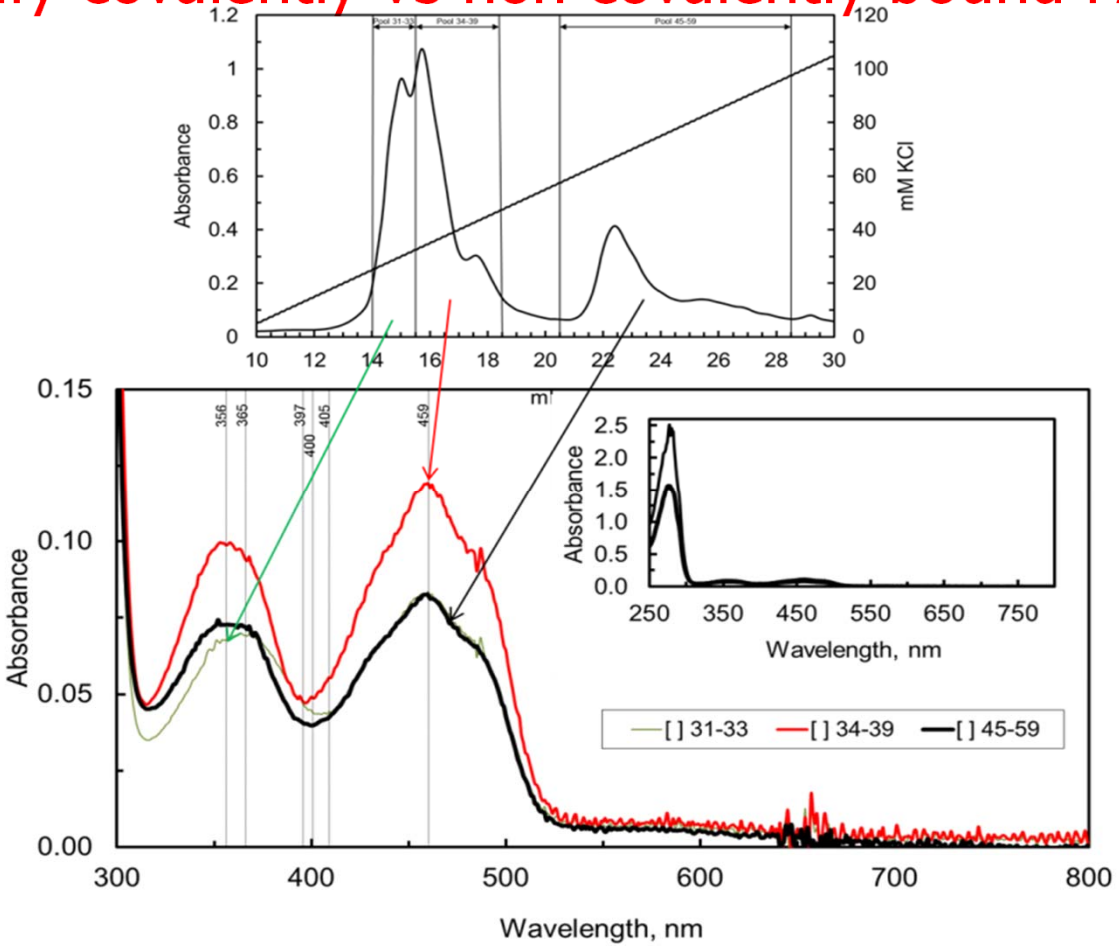
Example: protein concentration determination in samples of DMGDH from MonoQ to determine FAD stoichiometry and to quantify covalently vs non covalently bound FAD

Apo-DMGDH

DMGDH●FAD
(non covalent)

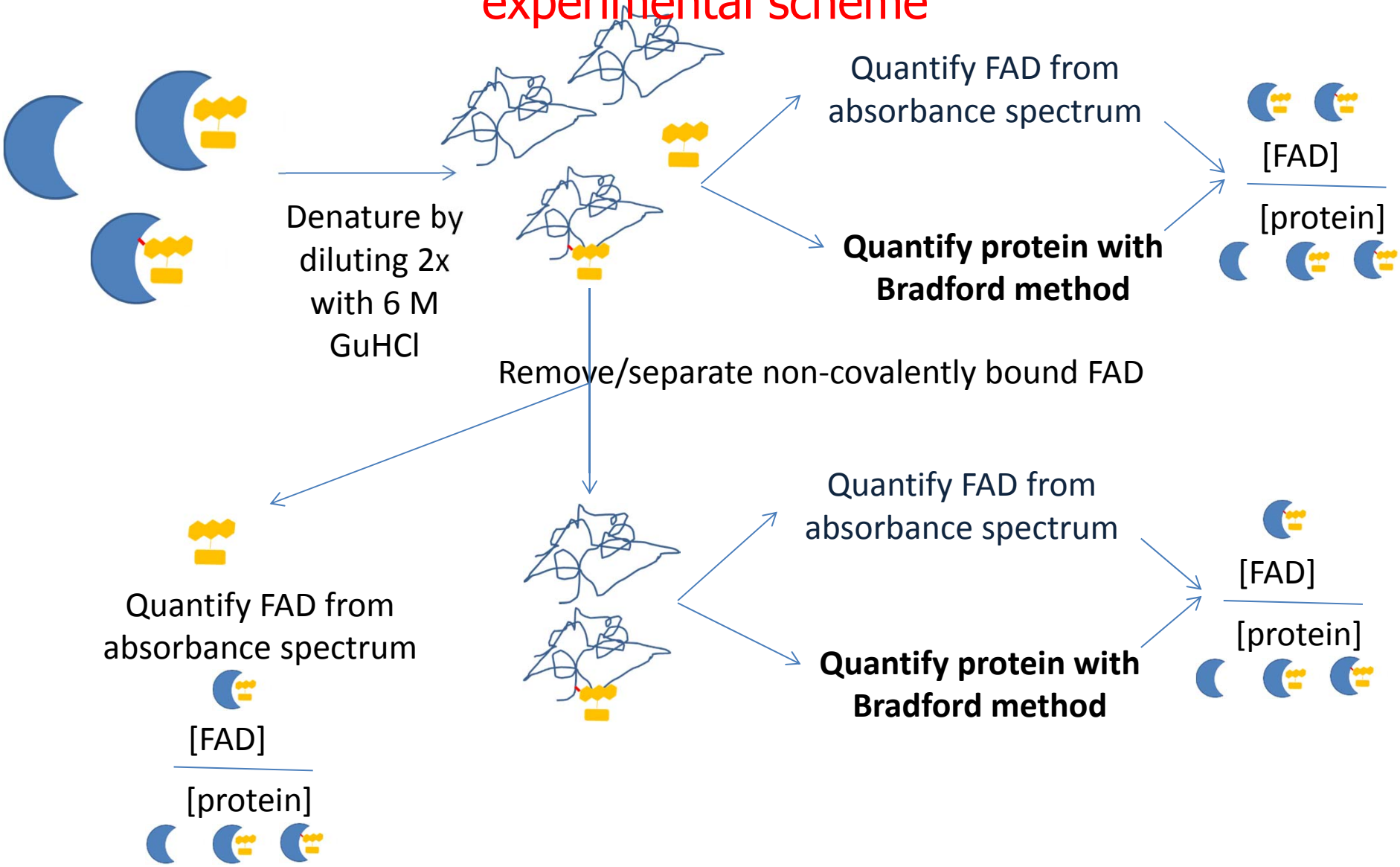
Holo-DMGDH
(covalently bound FAD)





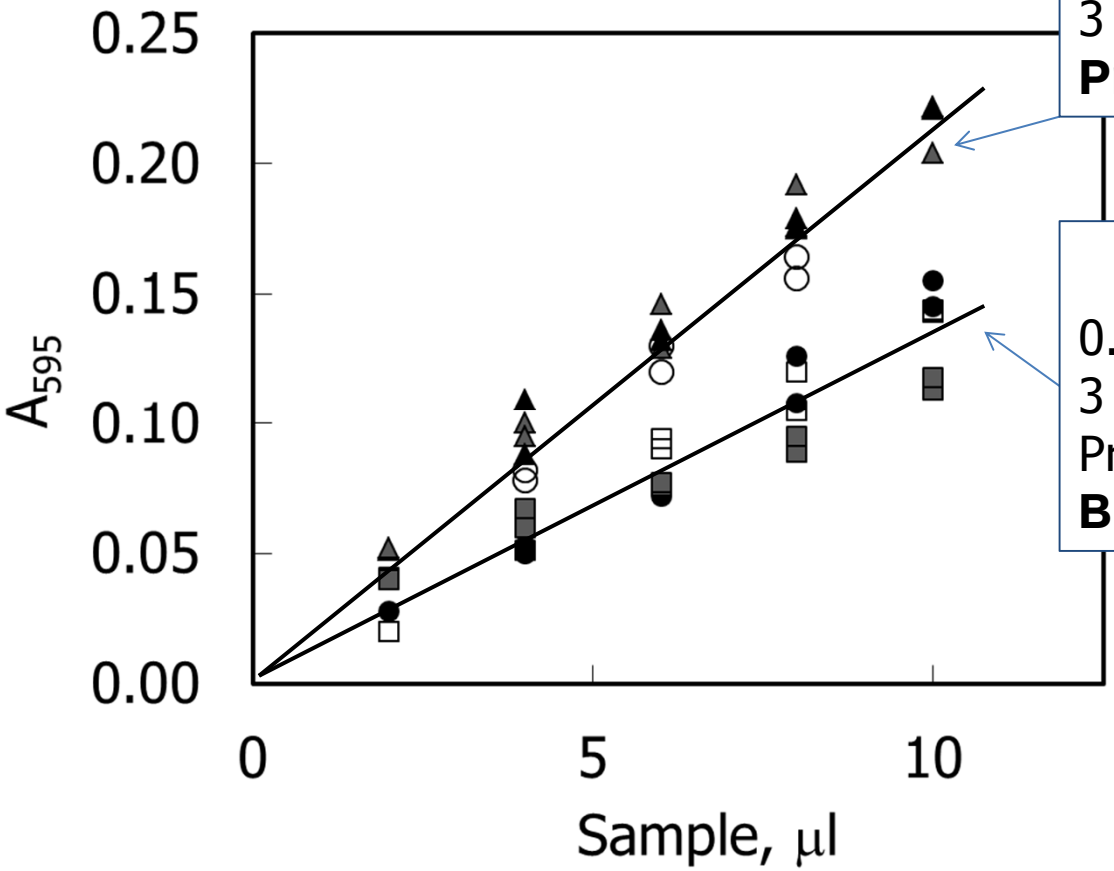
Spectra are similar at 459 nm, but different in the 360 nm region suggesting different forms

Example: protein concentration determination in samples of DMGDH from MonoQ to determine FAD stoichiometry and to quantify covalently vs non covalently bound FAD: the experimental scheme



Bradford Assay with DMGDH samples in 3 M Gu/HCl (30 mM GuHCl in assay – constant) : sensitive to the order of addition of reagents (due to protein precipitation ?).

No such problem with BCA assay



Assay method B

Bradford reagent	1 ml
0.1 M NaCl :	90 ul
3 M Gu/HCl buffer	(10 – x) ul
Protein solution	**x ul**

Assay method A

0.1 M NaCl :	90 ul
3 M Gu/HCl buffer	(10 – x) ul
Protein solution	x ul
Bradford reagent	**1 ml**

From MariangelaCamozzi thesis

Protein integrity

- SDS-PAGE (proteolytic degradation but sometimes anomalous migration)
- N-terminal sequencing
- MALDI-TOF (Matrix Assisted Laser Desorption/Ionization-Time of flight)

(Optimization of) Protein stability

- Activity assays with protein preincubated under various conditions
- Thermofluor
- DLS

Reasons for anomalous behaviour in SDS-PAGE

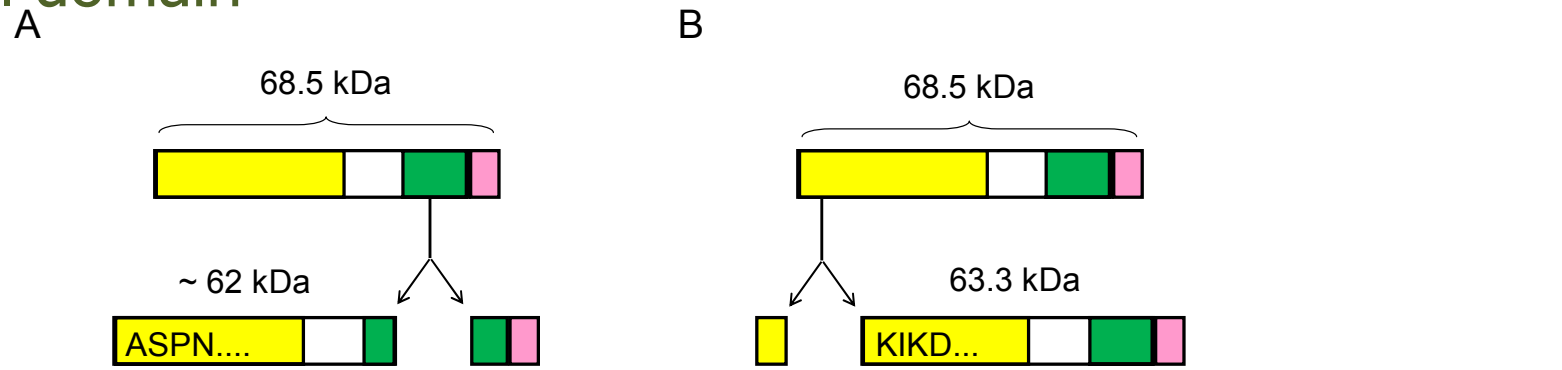
SDS/protein ratio different from 1.4 g SDS/g protein due to :
Electrostatics attraction/repulsion
Hydrophobic patches
Residual 3D structure

Altered charge/mass ratio due to protein positive charge

Typically slower migration for glycoproteins, acidic proteins, basic proteins but some exceptions may be found

Example: MICAL-MOCH (pI 7-7.5)

In SDS-PAGE MICAL-MOCH showed a mass (62 kDa) lower than the expected one (68.5 kDa). Mass spectrometry after tryptic digestion led to the conclusion that the protein lacked part of the N-terminal domain




```
     1          11         21         31         41         51         61         71         81         91
   1 ASPTSTNPAH AHFESFLQAQ LCQDVLSSFQ ELCGALGLEP GGGLPQYHKI KDQLNYWSAK SLWTKLDKRA GQPVYQQGRA CTSTKCLVVG AGPCGLRVAV
 101 ELALLGARVV LVEKRTKFSR HNVLHLPFTI HDLRALGAKK FYGRFCTGTL DHISIRQLQL LLLKVALLLG VEIHWGVTFT GLQPPPRKGS GWRAQLQPNP
 201 PAQLANYEFD VLISAAGGKF VPEGFKVREM RGKLAIGITA NFVNGRTVEE TQVEISGVAR IYNQSFFQSL LKATGIDLEN IVYYKDDTHY FVMTAKKQCL
 301 LRLGVLRQDW PDTNRLLGSA NVVPEALQRF TRAAADFATH GKLGKLEFAQ DAHGQPDVSA FDFTSMMRAE SSARVQEKHG RLLLGLVGDC LVEPFWPLGT
 401 GVARGFLAAF DAAWMVKRWA EGAESLEVLA ERESLYQLLS QTSPENMHRN VAQYGLDPAT RYPNLNLRAV TPNQVRDLYD VLAKEPVQRN NDKTDTGMPA
 501 TGSAGTQELL RWCQEQTAGY PGVHVSDLSS SWADGLALCA LVYRLQPGLL EPSELQGLGA LEATAWALKV AENELGITPV VSAQAVVAGS DPLGLIAYLS
 601 HFHSAFKSMA LEHHHHHH
```

N-terminal sequencing, MALDI-TOF, mass spectrometry after chymotryptc treatment revealed that the protein was actually intact!

Teresa Vitali, 2012

Protein integrity

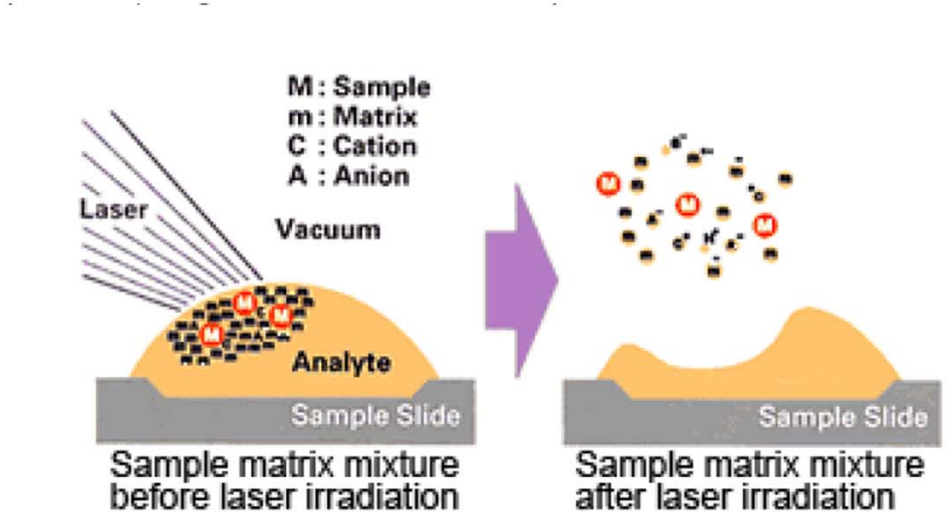
- SDS-PAGE (proteolytic degradation but also anomalous migration)
- N-terminal sequencing
- MALDI-TOF (Matrix Assisted Laser Desorption/Ionization-Time of flight)
- Proteolytic digestion + mass spectrometry

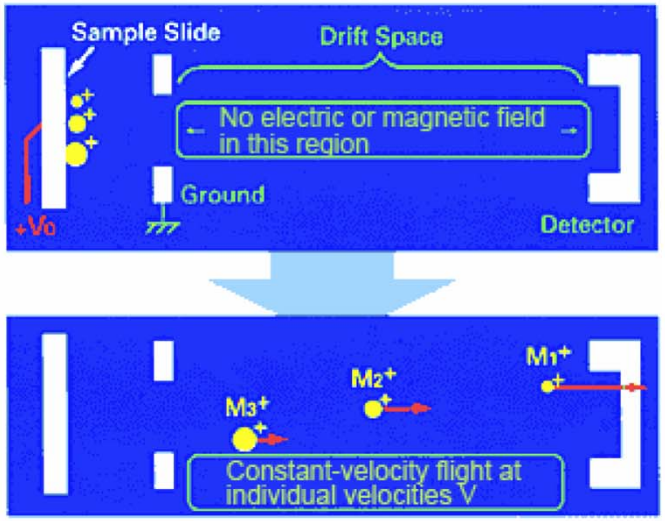
(Optimization of) Protein stability

- Activity assays with protein preincubated under various conditions
- Thermofluor
- DLS

- Limited proteolysis to determine flexible regions and conformationa changes

MALDI-TOF : the principle





Sample preparation:

- high protein concentration but low amount (as low as 0.5 pmol) in low concentration buffer/salts;
- protein concentration/buffer exchange through precipitation and resolubilization and/or (e.g.) spun columns

Select matrix (will the protein fly?)

Example in

jove Journal of Visualized Experiments www.jove.com

Video Article

Matrix-assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) Mass Spectrometric Analysis of Intact Proteins Larger than 100 kDa

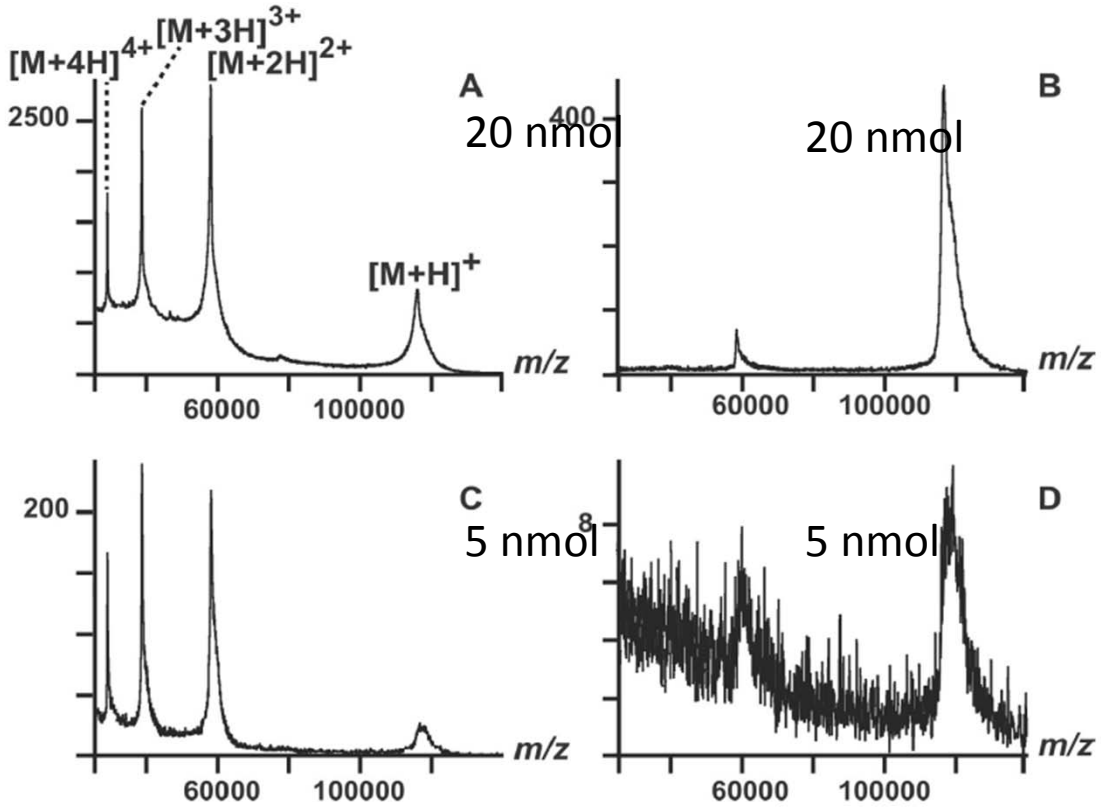
Luca Signor[1], Elisabetta Boeri Erba[1]

[1]Institute of Structural Biology "J.P. Ebel", UMR5075, Commissariat à L'Energie Atomique et aux Energies Alternatives (CEA), Centre National de la Recherche Scientifique (CNRS), Université J. Fourier

Correspondence to: Elisabetta Boeri Erba at eboerierba@googlemail.com

URL: http://www.jove.com/video/50635
DOI: doi:10.3791/50635

Keywords: Chemistry, Issue 79, Chemistry Techniques, Analytical, Mass Spectrometry, Analytic Sample Preparation Methods, biochemistry,



Test protein:
β-galactosidase

Different matrices

2,5-dihydroxybenzoic acid (DHB) +
α-cyano-4-hydroxycinnamic acid (α-CHCA)

sinapinic acid (SA)

_CHCA. **B)** amount: 20 pmoles;

Protein integrity

- SDS-PAGE (proteolytic degradation but also anomalous migration)
- N-terminal sequencing
- MALDI-TOF (Matrix Assisted Laser Desorption/Ionization-Time of flight)

(Optimization of) Protein stability

- Activity assays with protein preincubated under various conditions
- Thermofluor
- DLS

Dynamic light scattering

Using DLS for Growing Crystals of a Macromolecule

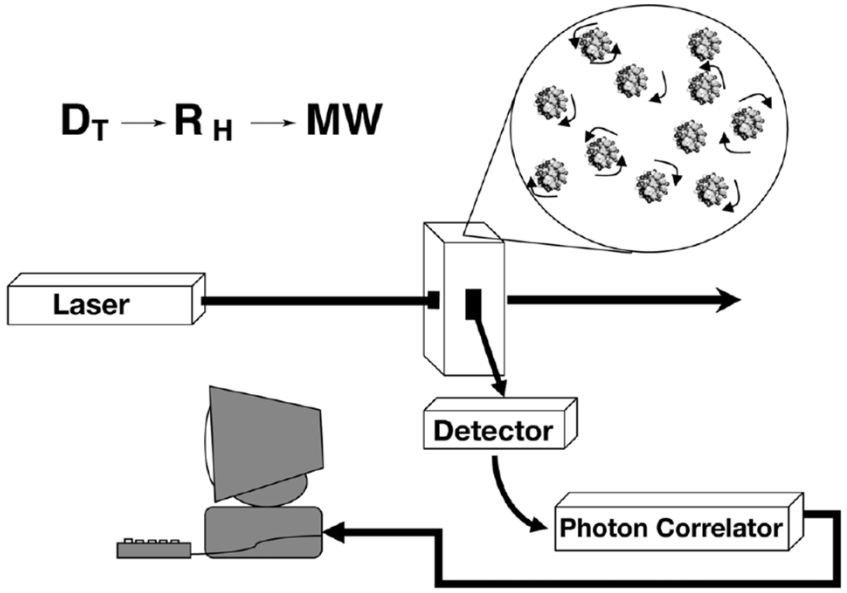
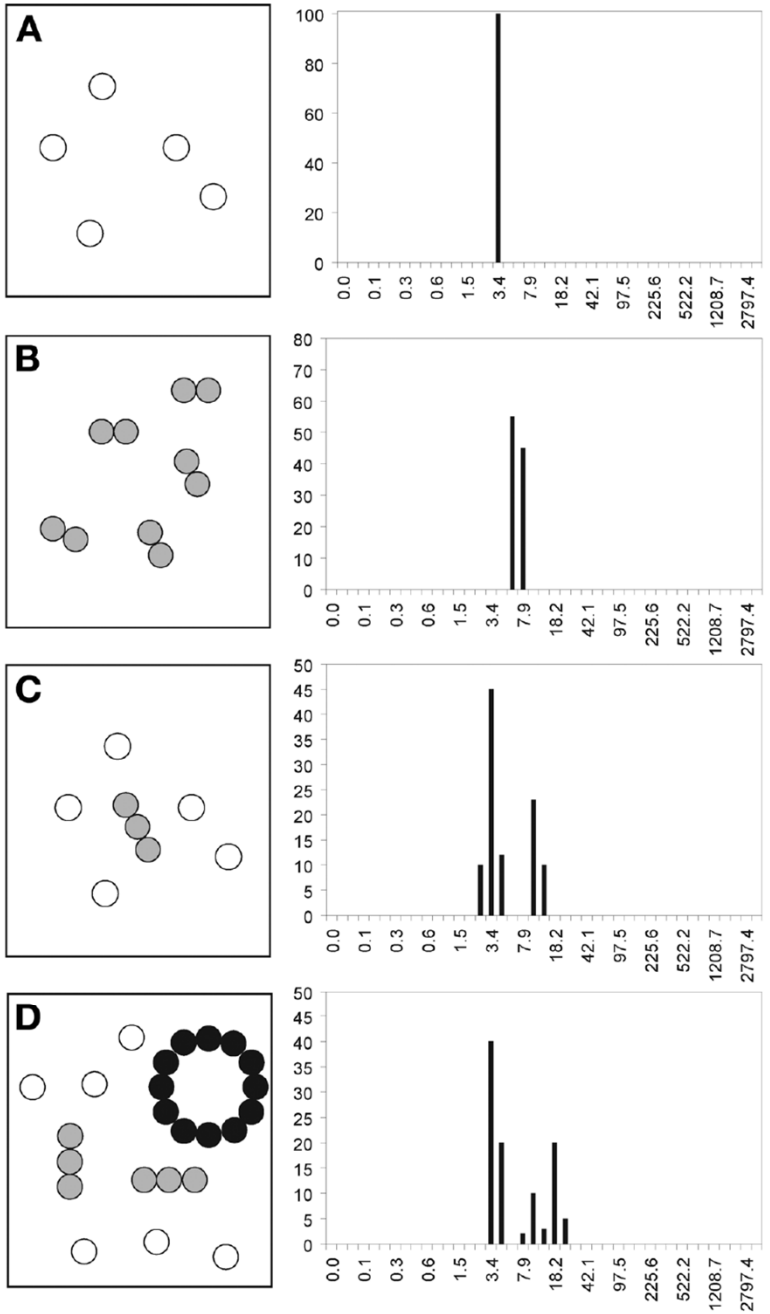


Fig. 2. Simplified schematic of the dynamic light scattering experiment.



Select your fractions off gel filtration to remove aggregates and select the desired(?) oligomeric state of the protein (?)

Biotechnology and Genetic Engineering Reviews - Vol. 24, 117-128 (2007)

Dynamic light scattering as a relative tool for assessing the molecular integrity and stability of monoclonal antibodies

ULF NOBBMANN[1*], MALCOLM CONNAH[1], BRENDAN FISH[2], PAUL VARLEY[2], CHRIS GEE[2], SANDRINE MULOT[2], JUNTAO CHEN[3], LIANG ZHOU[3], YANLING LU[3], FEI SHENG[3], JUNMING YI[3] AND STEPHEN E. HARDING[3]

[1]Malvern Instruments Ltd, Grovewood Road, Malvern, WR14 1XZ, UK, [2]Cambridge Antibody Technology, Milstein Building, Granta Park, Cambridge, CB1 6GH, UK and [3]School of Biosciences, University of Nottingham, Sutton Bonington, LE12 5RD, UK

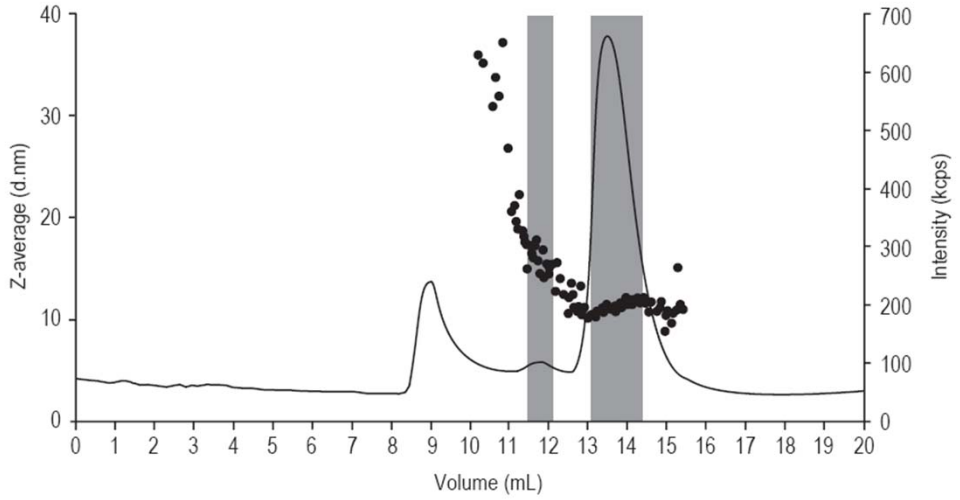


Figure 4. Chromatogram of the purified antibody Ab3. Intensity (grey line) and hydrodynamic diameter (black symbols) versus elution volume. The main peak is the monomer, with some aggregation visible in the void volume and a minor component from dimer. The monomer shows a high intensity with a relatively constant size during the elution of the peak.

DLS: change buffers, temperature and protein concentration to determine/optimize stability

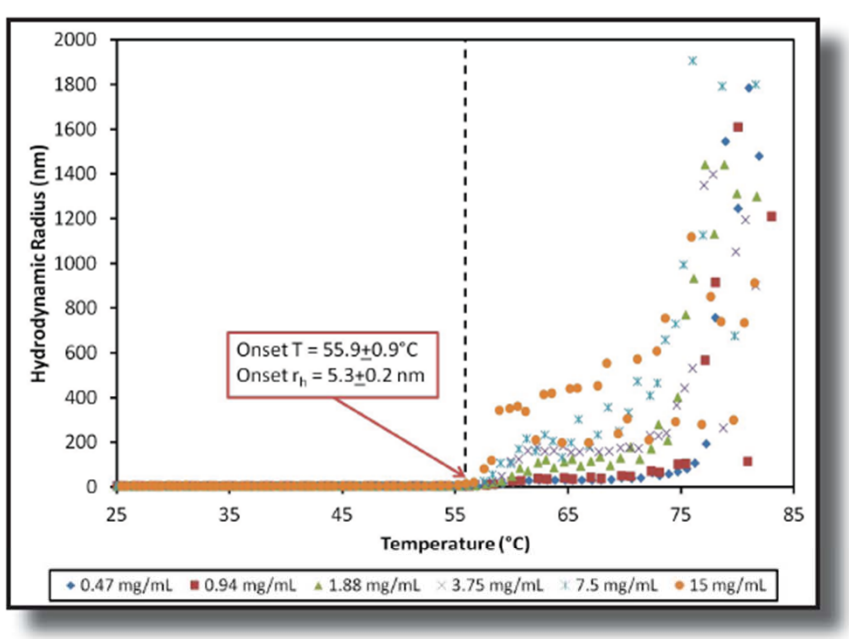


Figure 1. Hydrodynamic radius as a function of temperature and concentration for an antibody formulation at pH 8.5. High order aggregate formation is evident for temperatures >56°C.

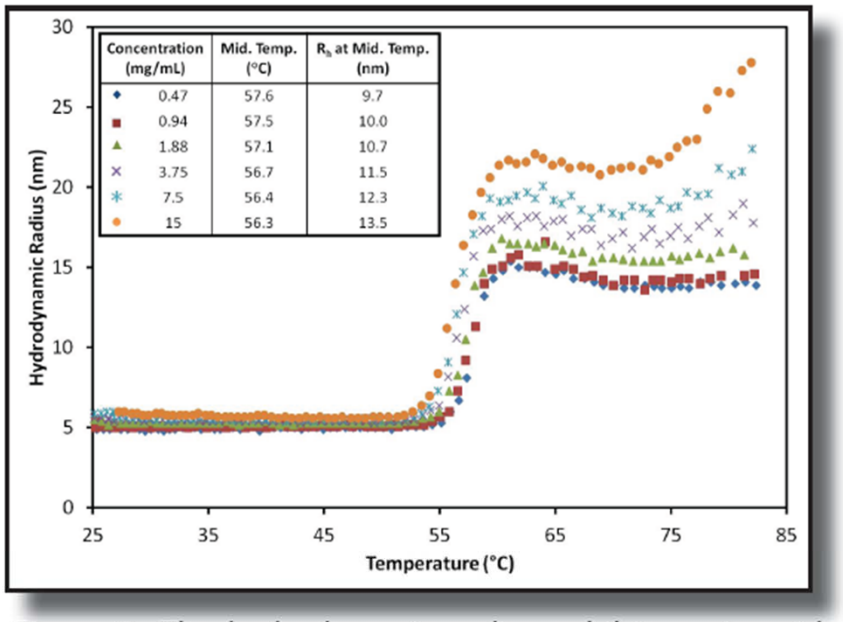
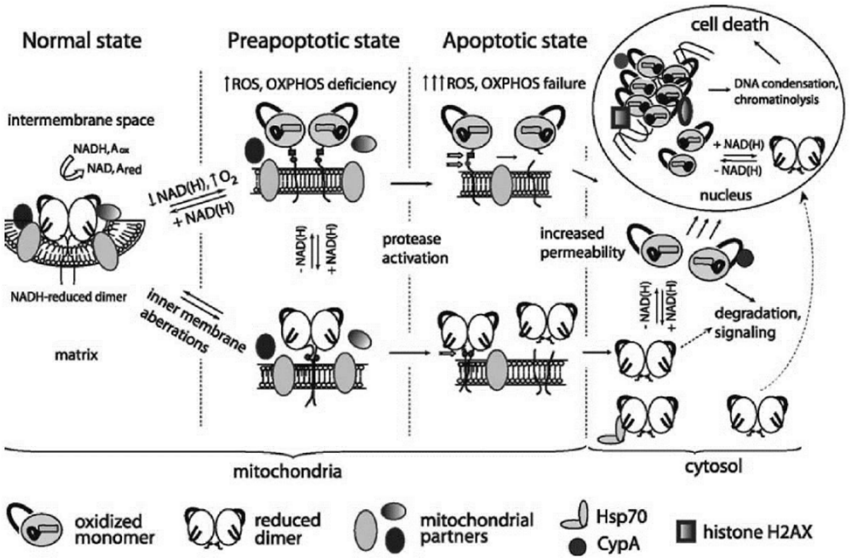


Figure 2: The hydrodynamic radius exhibits a sigmoid relationship as a function of temperature for all antibody concentrations at pH 9.5. The change in midpoint temperature and radius as a function of concentration may indicate nonspecific attraction or oligomerization.

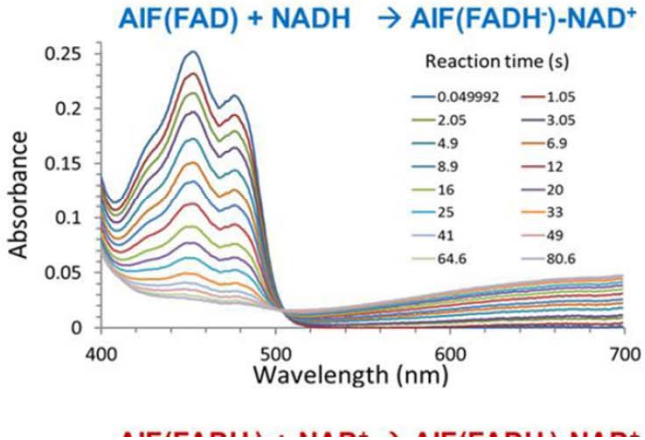
Use DLS to follow kinetics of association/dissociation of protein:

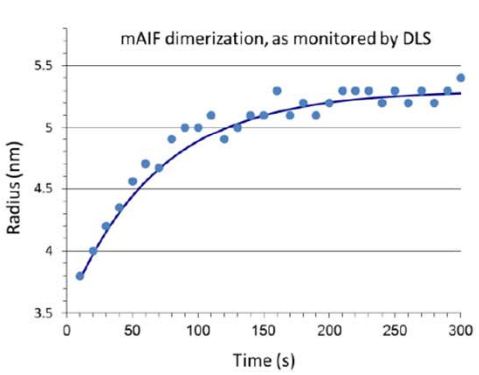
Apoptosis Inducing factor (AIF): Flavoprotein anchored to the inner mitichondrial membrane, facing the intermembrane space: unknown catalytic activity (if any) but essential for maintenance of Complex I and II (?); moonlights as apoptosis inducing factor. Very stable NADH reduced form (dimeric): a NADH sensor?

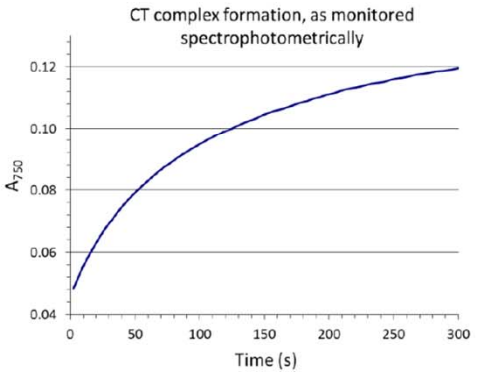


Presumed redox-sensing role of AIF. In healthy mitochondria, AIF exists as a reduced charge-transfer dimeric complex that contributes to OXPHOS functionality. Redox alterations and/or NAD(H) depletion promote AIF oxidation and dimer dissociation, which in in conjunction to other signals results in AIF release from mitochondria and apoptosis. Redox-linked dimerization of soluble AIF can prevent its apoptotic action providing another checkpoint of the apoptotic pathway. Taken from Sevrioukova (2011) Antioxid Redox Signal 14, 2545-2579.

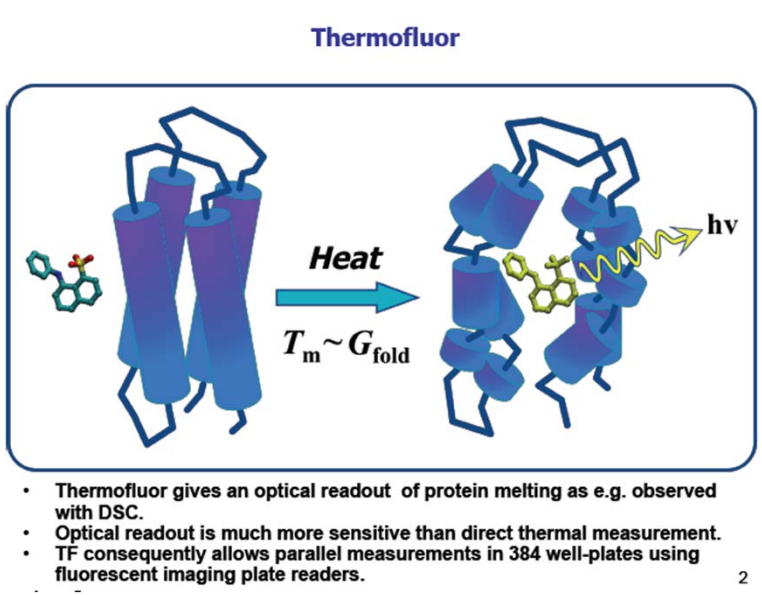
Monitor the dimerization of AIF upon reduction with NADH by DLS

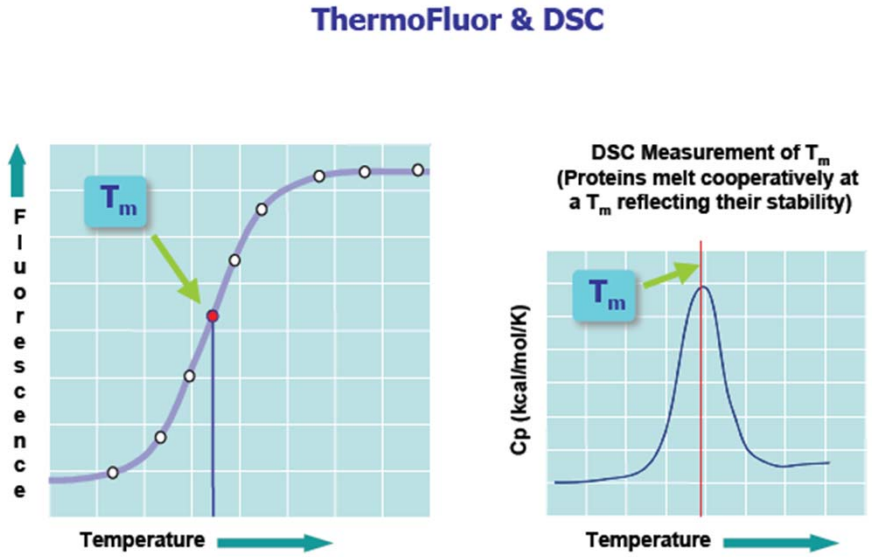




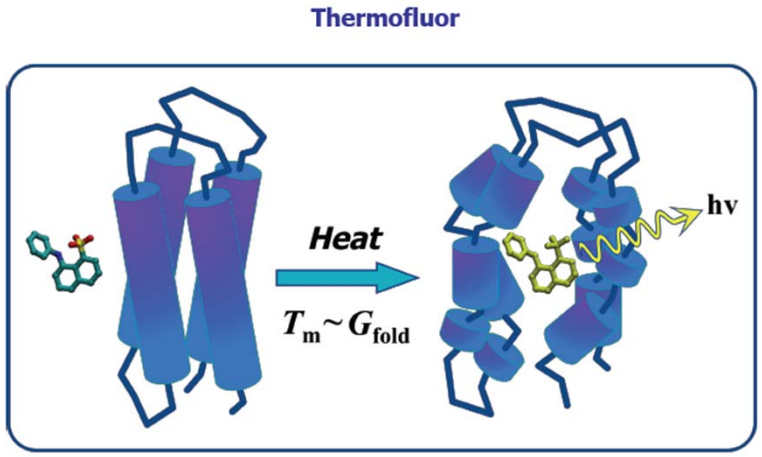


Thermofluor is easier than Differential scanning calorimetry. It is a combination of old and new technologies (Fluorescence emission enhancement of bound vs free fluorophor + real-time PCR technology





Several Dyes can be used



- **Thermofluor gives an optical readout of protein melting as e.g. observed with DSC.**
- **Optical readout is much more sensitive than direct thermal measurement.**
- **TF consequently allows parallel measurements in 384 well-plates using fluorescent imaging plate readers.**

2

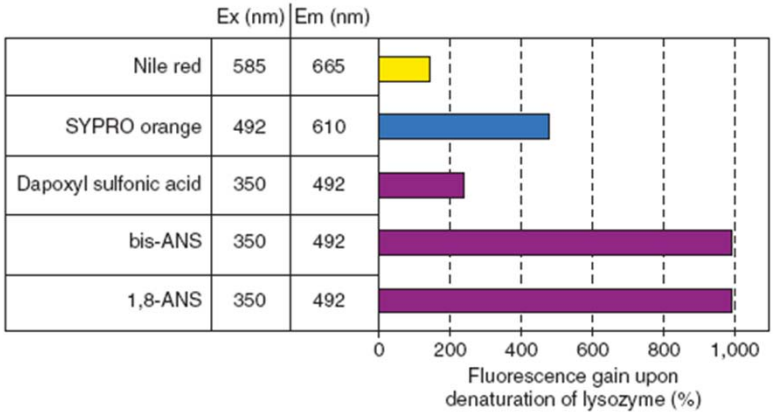
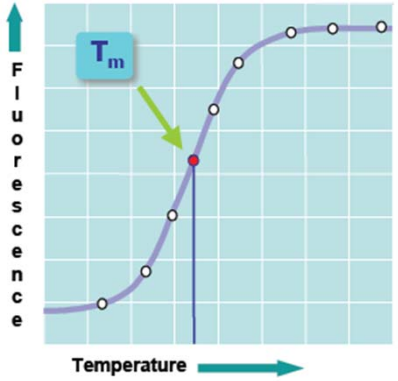
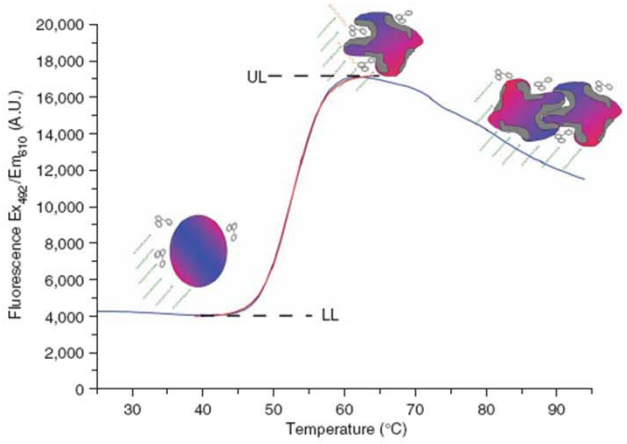


Figure 1 | Optical properties and fluorescence signal in the presence of lysozyme (native versus denatured) for selected dyes that can be used for DSF. All dyes (at 43 μM except SYPRO orange, which was diluted 1:250) were in a solution of 75 μg ml^{-1} hen egg lysozyme in buffer (10 mM 3-(cyclohexylamino)-1-propanesulfonic acid pH 9.0, 150 mM NaCl). The graph shows the difference in fluorescence intensity before and immediately after incubation for 5 min at 100 °C, respectively. The excitation and emission wavelengths given in the table refer to the custom filters for the Stratagene Mx3005p instrument.

The theoretical vs the actual trace

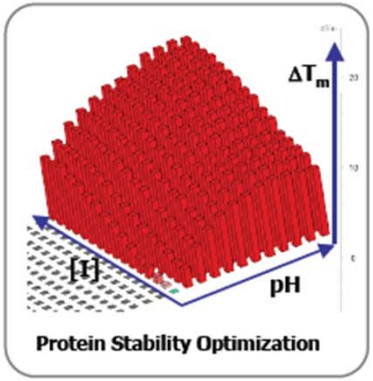




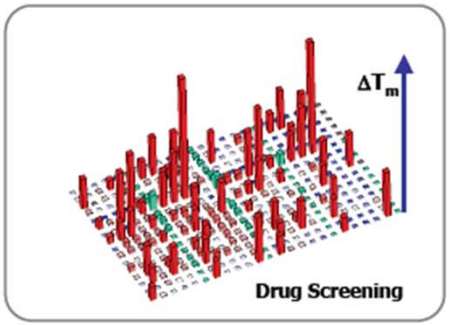
of over 2,000 compounds dissolved, whereas for 85% the solubility was 50 mM or higher. For long-term storage of light-sensitive compounds, brown glass vials with DMSO-resistant lids and seals

Figure 2 | Typical recording of fluorescence intensity versus temperature for the unfolding of protein (citrate synthase) in the presence of SYPRO orange. The dye, the molecular structure of which is undisclosed, is symbolized as a three-ring aromatic molecule. In the presence of a globular protein (spherical shape at the baseline of the curve), a basic fluorescence intensity is excited by light of 492 nm (depicted schematically by green curved arrows). Through unfolding of the protein, hydrophobic patches (in gray) become exposed, and strong fluorescent light of 610 nm (depicted by orange curved arrows) is emitted by the dye molecules bound to them. Following the peak in the intensity, a gradual decrease is observed, which is mainly explained by protein being removed from solution owing to precipitation and aggregation. The lower and upper level in the fluorescence intensity, LL and UL, respectively, defined by equation (1), are depicted in the figure.

Thermofluor Applications

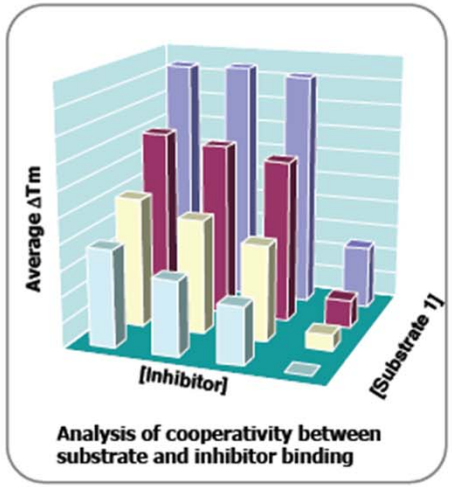


Protein Stability Optimization



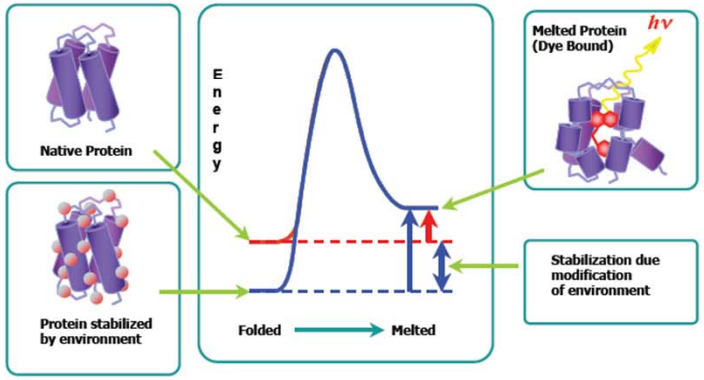
Drug Screening

www.thermofluor.org

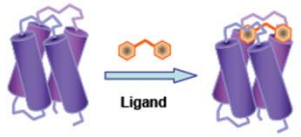


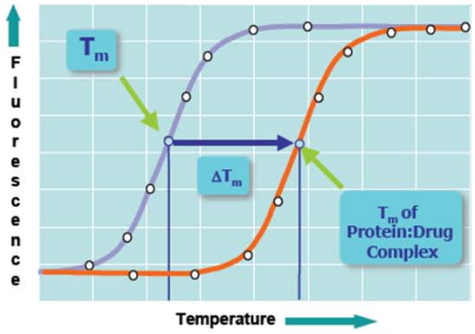
Analysis of cooperativity between substrate and inhibitor binding

Thermofluor Can Measure Protein Stabilization



Ligand Binding Can Stabilize Protein Structure





Journal of Biomolecular Screening
http://jbx.sagepub.com/

Enhancing Recombinant Protein Quality and Yield by Protein Stability Profiling

Tara M. Mezzasalma, James K. Kranz, Winnie Chan, Geoffrey T. Struble, Céline Schalk-Hihi, Ingrid C. Deckman, Barry A. Springer and Matthew J. Todd

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DOI: 10.1177/1087057106297984

The online version of this article can be found at:
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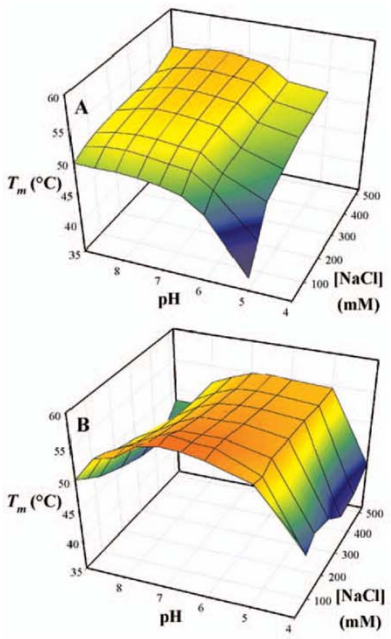


FIG. 2. Protein stability surfaces. A subset of *ThermoFluor*®-derived T_m values from the pH-salt profile plotted as a function of NaCl and pH, generating a stability surface for (**A**) cFMS and (**B**) Akt-3. Stability surfaces represent 9 buffers (acetate, pH 4 and 5; MES, pH 6 and 6.5, HEPES, pH 7, 7.5, and 8; borate, pH 8.5; each at 25 mM) and 7 [NaCl] (25, 50, 100, 200, 300, 400, and 500 mM).

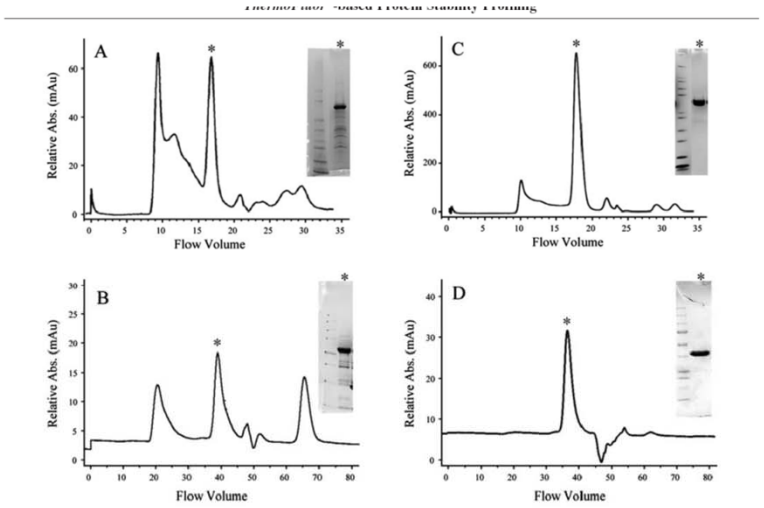


FIG. 5. Protein aggregation and purity before and after protein stability profiling (PSP)–optimized conditions. Size exclusion chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (insets) for cFMS (**A, C**) or AKT3 (**B, D**) from kinase purifications before (**A, B**) and after (**C, D**) optimization; the asterisk denotes monomer fractions analyzed by SDS-PAGE. (**A**) cFMS protein purified from Ni-NTA column, showing aggregation; purity was ~90% by SDS-PAGE. (**B**) Akt-3 protein purified by Ni-NTA column showing contamination and aggregation; monomer purity was < 90% by gel analysis. (**C**) Optimized cFMS protein; purity was >98% by SDS-PAGE. (**D**) Optimized Akt-3; purity was >98% by SDS-PAGE.

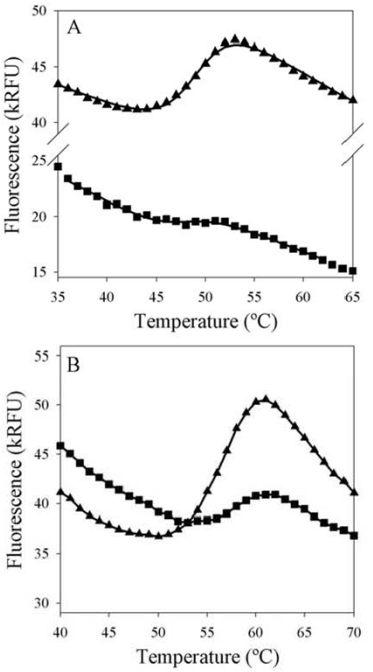


FIG. 6. Relative fluorescence from aggregate-prone protein (squares) versus protein from optimized purification procedures (triangles). (**A**) 50 µg/mL cFMS, 25 µM ANS, 100 mM NaCl, 25 mM HEPES, pH 7.0. (**B**) 150 µg/mL Akt-3, 50 µM ANS, 100 mM NaCl, 25 mM HEPES, pH 7.0.

Bottom line: The optimization of buffer composition during purification leads to higher yields and higher quality protein preparations

Example: Use Thermofluor to characterize/stabilize HIV1 protease monomeric variant to use to study folding/unfolding with molecular tweezers: introduce substitutions to obtain monomer and Cys at N and C-terminus to attach DNA handles

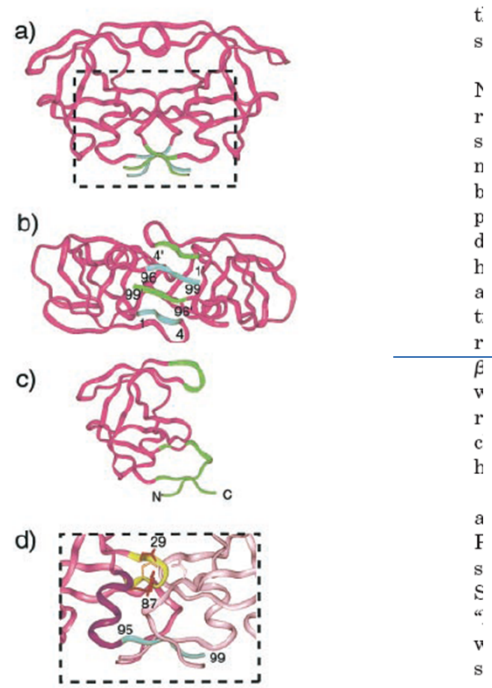
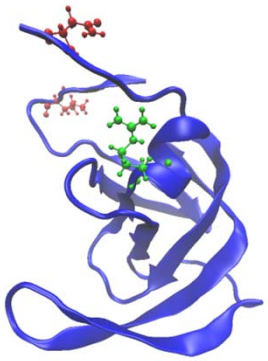
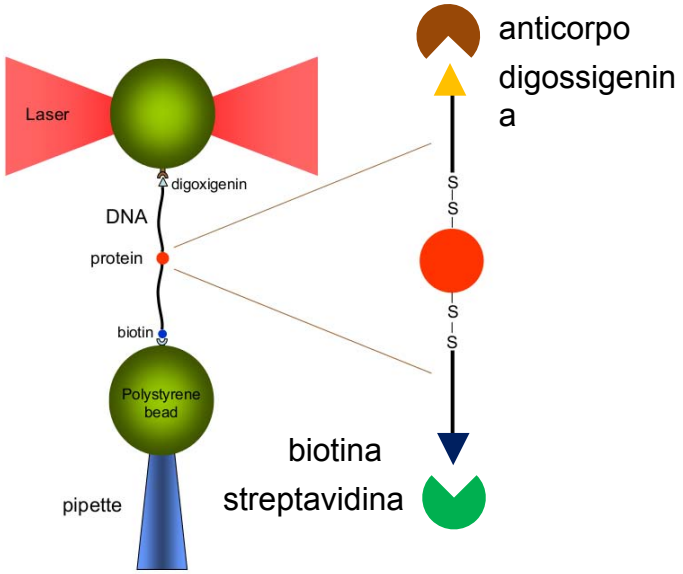


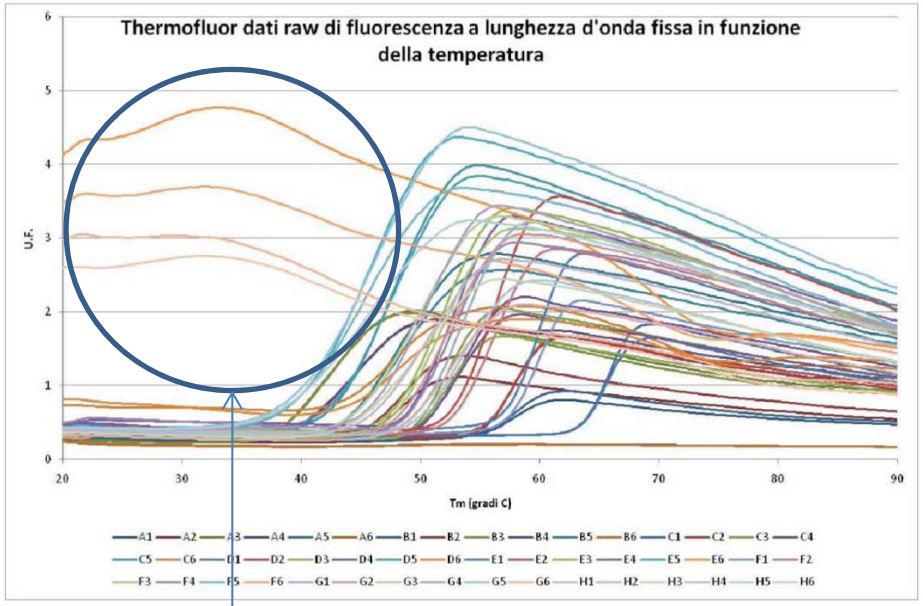
FIG. 4. **Ribbon representations of the structure of mature HIV-1 protease (*a*), the terminal β-sheet interface (*b*), regions that exhibit pico/nanosecond time scale motions (NOE < 0.5) in the PR_{R87K} monomer (shown in *green*) (*c*), and details of the structure surrounding Arg^{87} (*d*).** In *a* and *b*, the terminal β-strands (residues 1–4 and 96–99) of one subunit are colored in *green* and the other *cyan*. In *d*, the side chains of Asp^{29} and Arg^{87} are depicted in *red*, and the side chains of the active-site Asp^{25} residues are colored in the same color as the corresponding subunit backbone. The region (residues 87–95) encompassing the α-helix is shown in *purple*, and the adjoining C-terminal β-strand is shown in *cyan* in *d*. The structures were drawn using the coordinates of Protein Data Bank code 1A8K (www.rcsv.org).



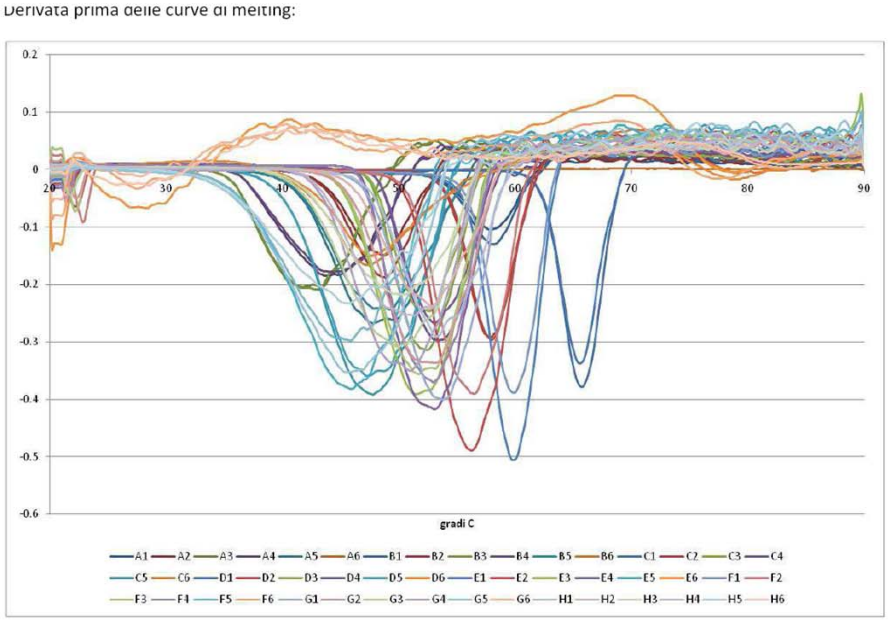


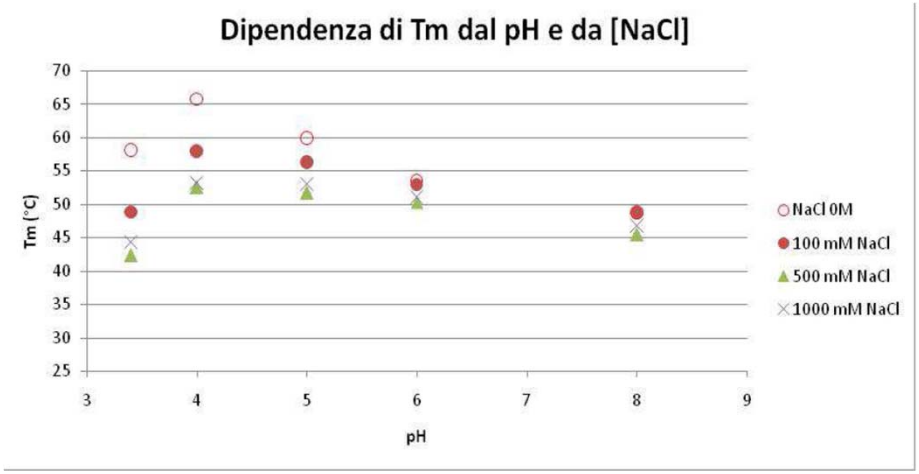
Martina Caldarini, G. Tiana Dept of Physics, UniMI; C. Cecconi, UniMOdena

Optimize protein concentration, then test different conditions with different protein forms



Denatured even at low temperature





Thermofluor: a combination of old and new technologies

the Jo

*Thermo*FAD, a *Thermofluor*®-adapted flavin *ad hoc* detection system for protein folding and ligand binding

Federico Forneris, Roberto Orru, Daniele Bonivento, Laurent R. Chiarelli and Andrea Mattevi

Department of Genetics and Microbiology, University of Pavia, Italy

Exploiting an intrinsic chromophor: the case of flavoproteins in which protein denaturation leads to loss of flavin fluorescence quenching

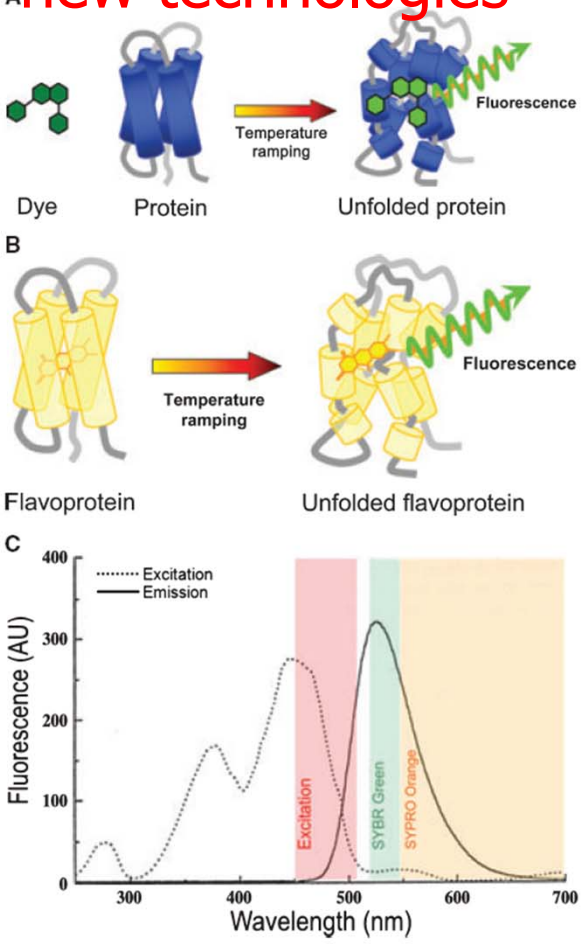


Fig. 1. (A) Schematic representation of the *Thermofluor*® binding assay. A solvatochromic dye (i.e. SYPRO Orange) is used as an indicator of protein unfolding. Binding of the dye to the unfolded protein results in a significant increase in its intrinsic fluorescence. (B) Schematic representation of *Thermo*FAD. In this case, the increase in fluorescence is generated by exposure of the flavin cofactor to the solvent upon protein unfolding. (C) Overview of fluorescence properties of flavins and comparison with RT-PCR instrumental parameters. Dashed line, flavin excitation spectrum; continuous line, flavin emission spectrum; red, wavelength range for RT-PCR fluorescence excitation; green, SYBR Green detection range; orange, SYPRO Orange detection range. Flavin fluorescence emission can be measured using the SYBR Green fluorescence filter on the RT-PCR instrument without any adaptation.

Comparing denaturation curves of protein in the absence/ presence of dye reveals that the dye may alter protein behavior

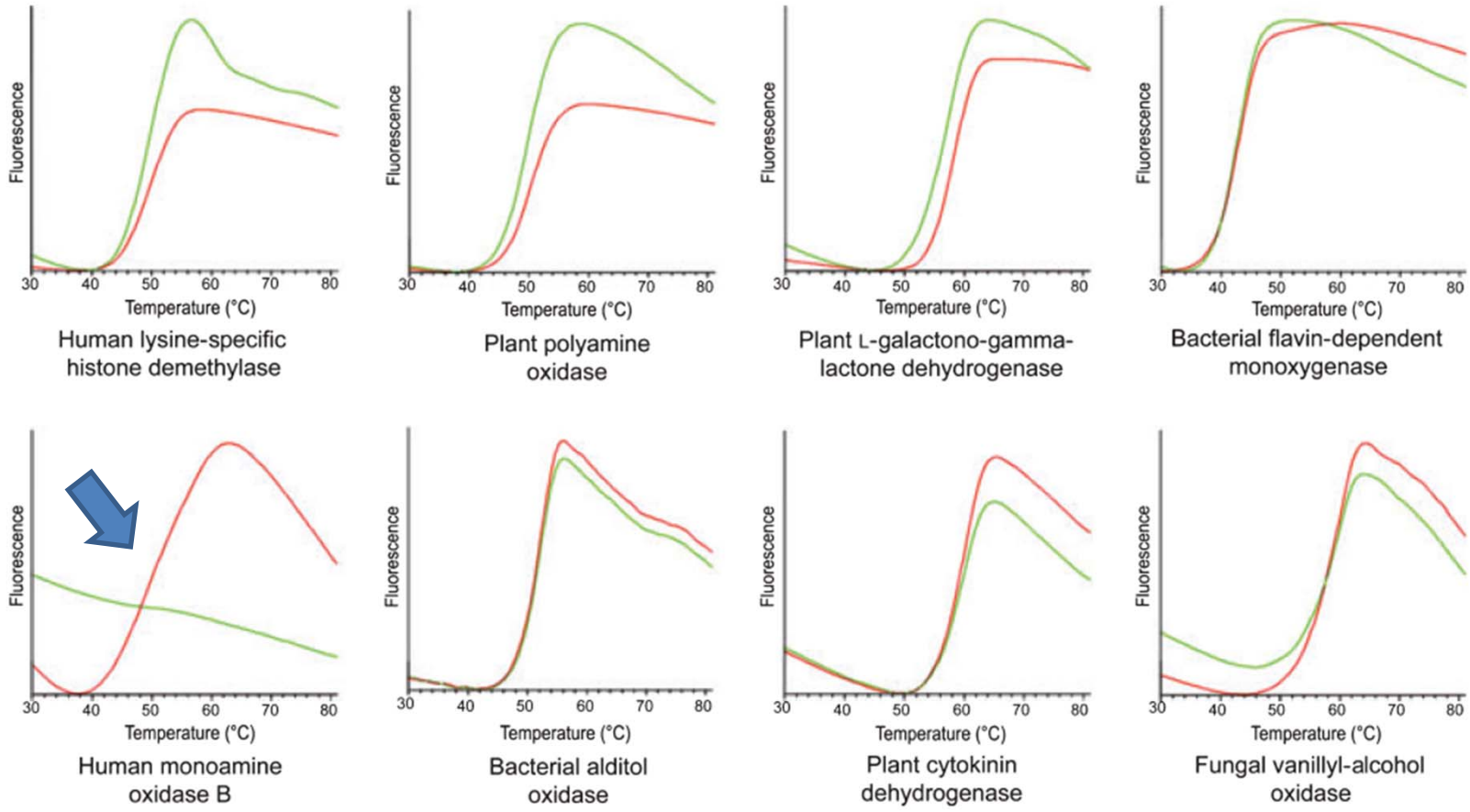


Fig. 2. Comparison between *Thermofluor*® and *Thermo*FAD for various flavoproteins. The selected flavoproteins differ with respect to the type of flavin cofactor, flavin linkage to the protein, and source organism of the protein (for details see Table 1). Thermal stability curves are plotted against normalised fluorescence signal. Green lines, *Thermofluor*® experiments using SYPRO Orange as fluorescent dye; red lines, *Thermo*FAD experiments measured without addition of any dye. The detector filter of the RT-PCR instrument for *Thermo*FAD is the one that is commonly used for SYBR Green dye (fluorescence emission of 523–543 nm; see Fig. 1C).

Color code: green, + SyproOrange; red , no SyproOrange

Sample preparation and characterization

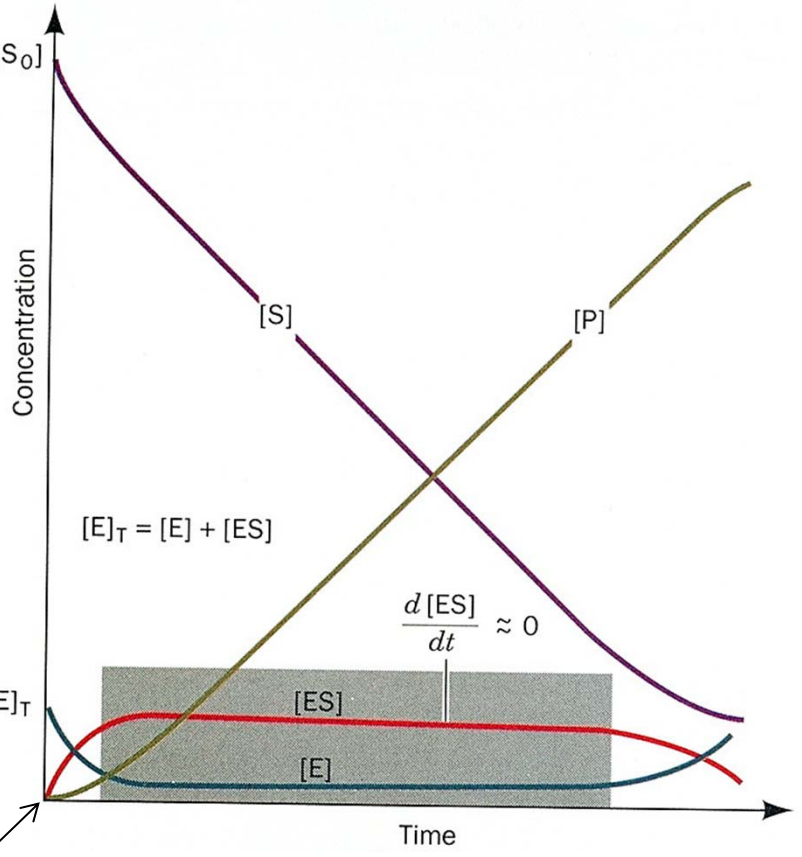
Structure-function studies require stable, reproducible, biologically active and homogeneous protein preparations.

Critical steps:

- Expression
- Purification
- Storage
- Protein concentration determination
- Cofactor/coenzyme content
- Chemical modifications
- Aggregation state
- Conformational changes
- Protein ligands
- Biological activity determination

Initial velocity measurements under steady-state conditions allow to determine the kinetic parameters V and K_M for the substrates, which depend on the rate constants that govern the individual reaction steps.

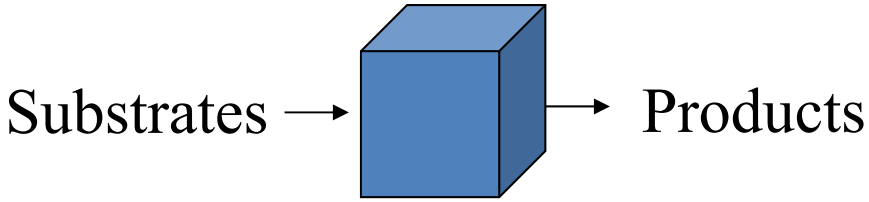
$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EP \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E+P$$



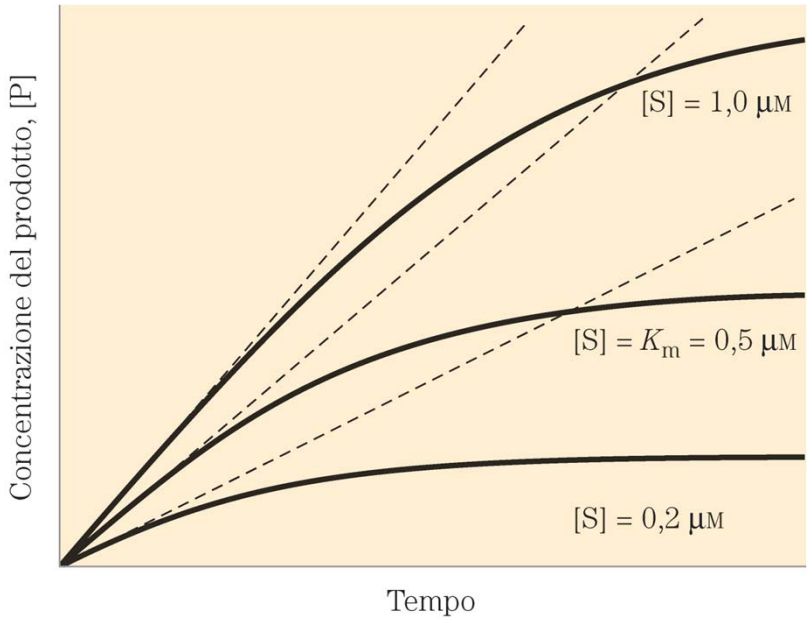
Velocity measurements under pre-steady-state conditions allow to determine directly the values of the rate constants that govern the individual reaction steps

Initial velocity measurements of the enzyme-catalyzed reaction are carried out, under a variety of conditions:

- to quantify the enzyme and
- to obtain information on the reaction mechanism, regulatory mechanisms, the active enzyme form.

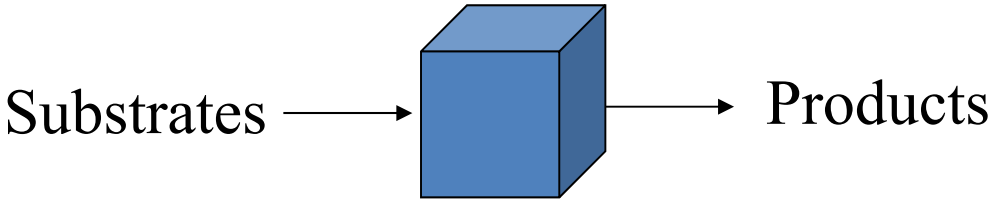


$$v = -\frac{d[S]}{dt} = \frac{d[P]}{dt}$$

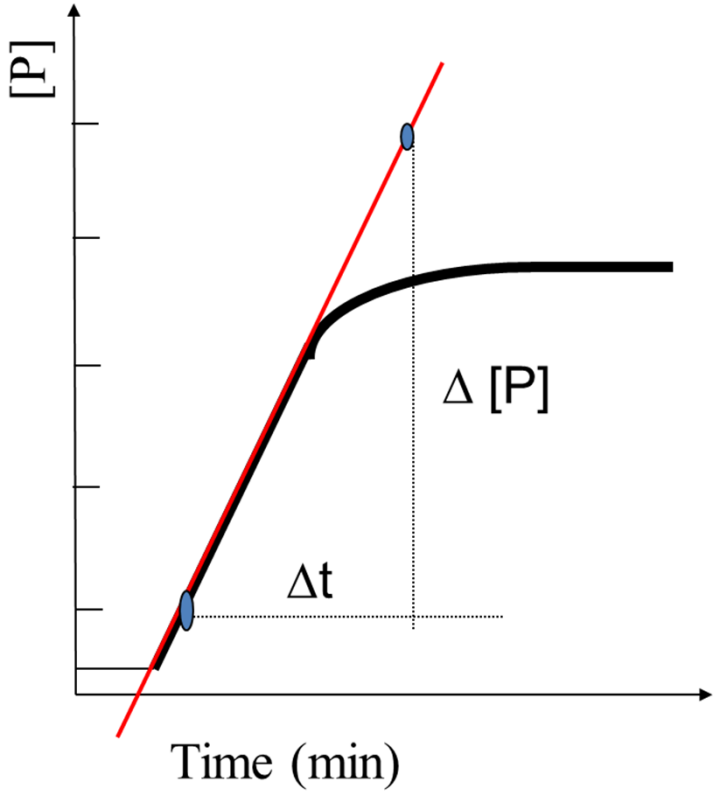


For enzymes:

- Reliable Activity assays
- Linearity of v vs [E]?
- Michaelis-Menten behaviour?
- Artifacts?
- Identification of coenzyme; stoichiometry



$$v = -\frac{d[S]}{dt} = \frac{d[P]}{dt}$$



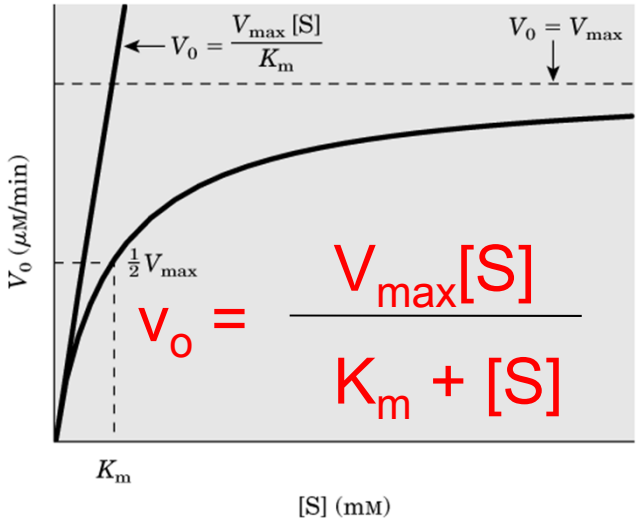
The Michaelis-Menten Equation.

for the Simple Model: $E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E+P$

$$v = \frac{k_3\,[Etot] * [S]}{\frac{k_2+k_3}{k_1} + [S]}$$

$$V_{max} = k_3[E_t]$$

$$K_m = \frac{k_2 + k_3}{k_1}$$



$$v_o = \frac{V_{max}[S]}{K_m + [S]}$$

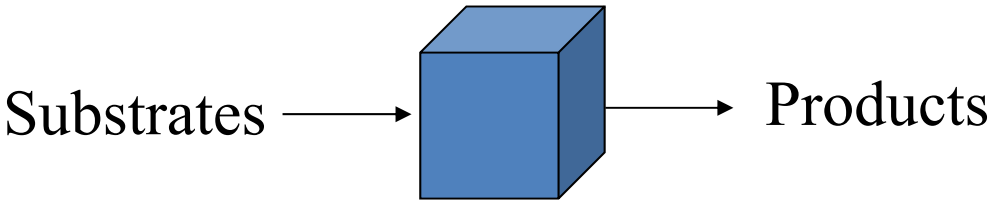
Simplifying....

k_1, k_2, k_3 will depend on , e.g., temperature; isotopic substitution of S and solvent; ionic strength of the medium, solvent viscosity

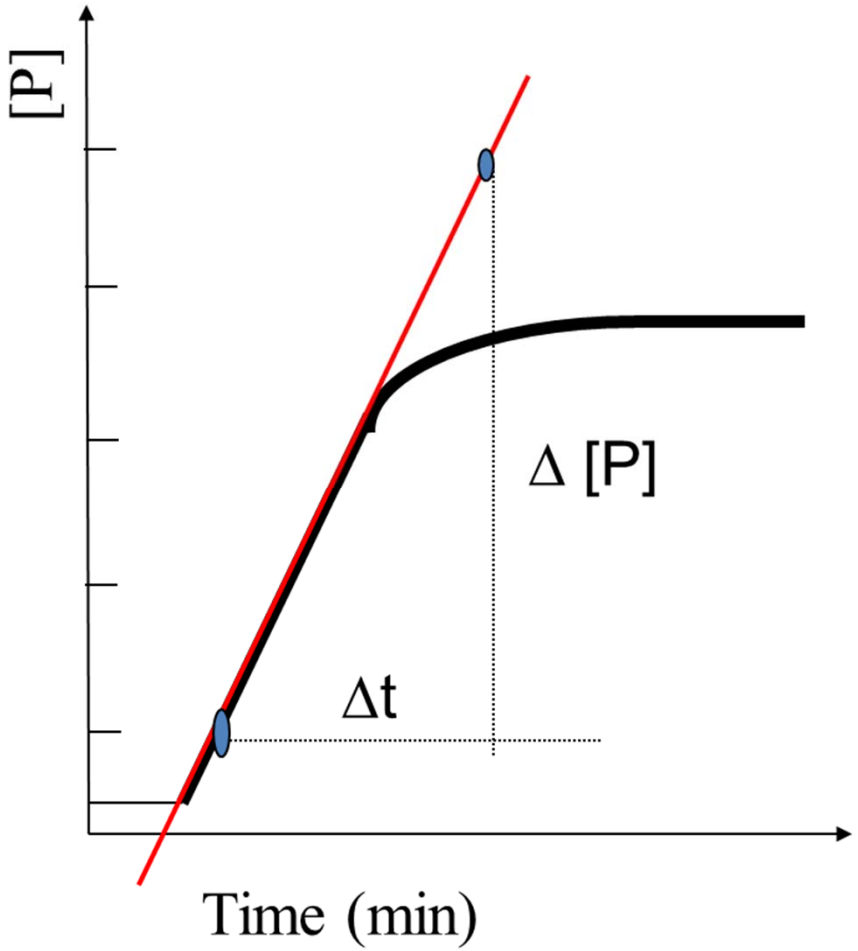
«Active» enzyme form with respect to S or P binding will depend on, e.g., pH, presence of inhibitors

Thus, from V and V/K values as a function of pH, I, etc will give information on E and the catalytic mechanism

Continuous spectrophotometric assays are very handy: no sample manipulation, direct observations, often high sensitivity, reproducibility.



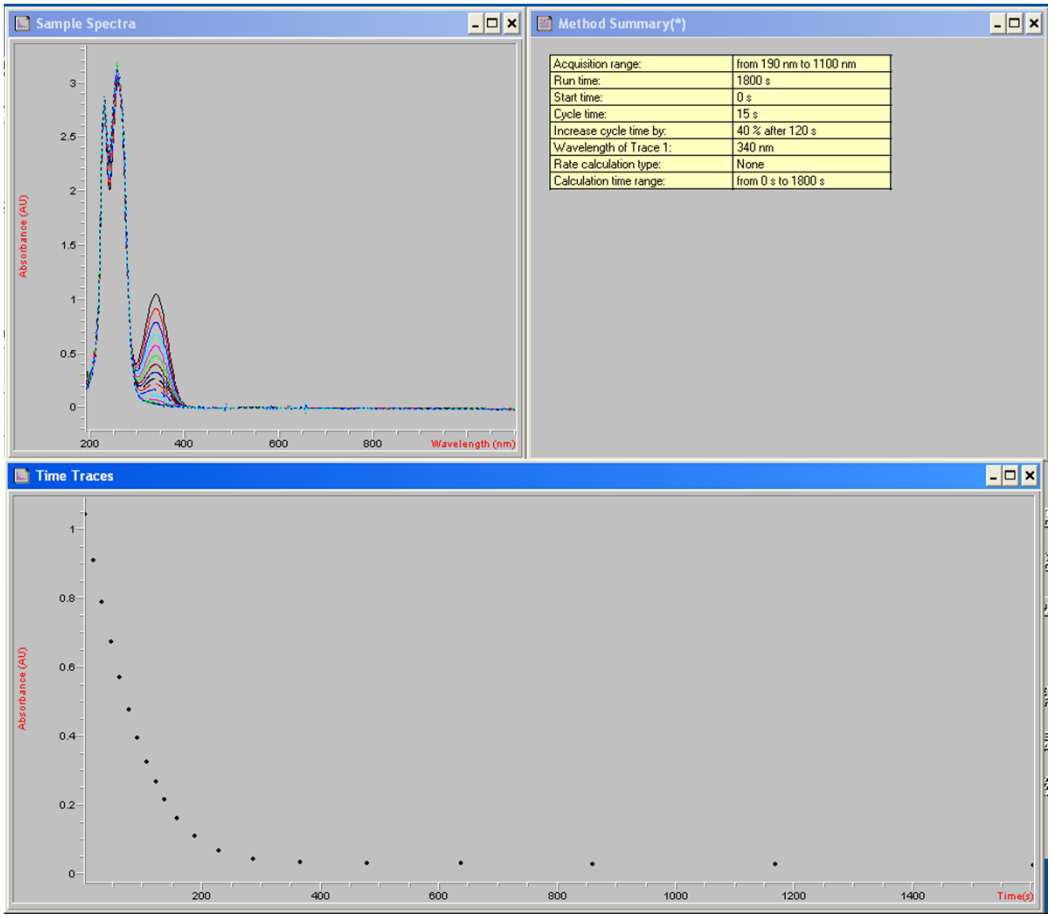
$$v = -\frac{d[S]}{dt} = \frac{d[P]}{dt}$$



Example:
Monitor NAD(P)H oxidation (or NAD(P) reduction) in reactions catalyzed by dehydrogenases/reductases

$$NADPH + H^+ + O_2 \xrightleftharpoons{\text{MICAL}} NADP^+ + H_2O_2$$

Monitoring the entire spectrum can help troubleshoot: aggregation , precipitation of substrates/products; artifacts



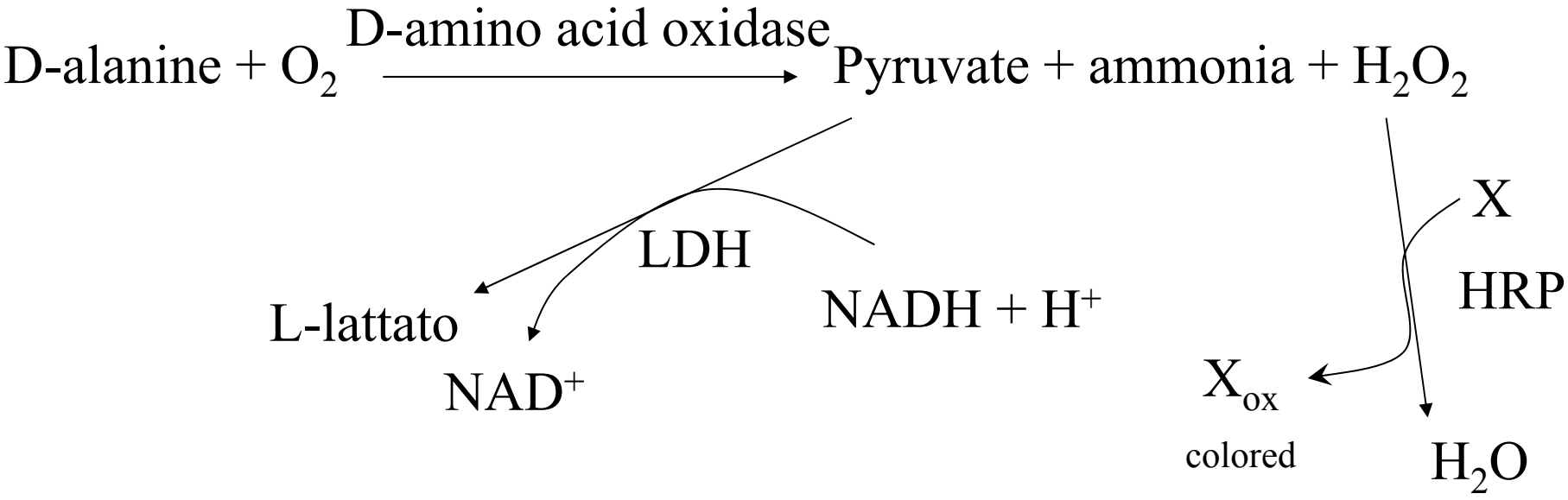
Coupling the reaction of interest with an indicator reaction with substrates/products suitable for a spectrophotometric assay is very handy.

For consecutive reactions:

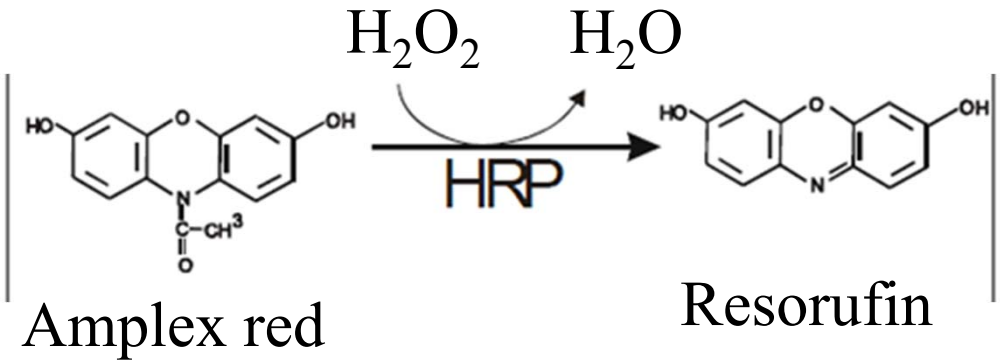
$$A \rightarrow B \rightarrow C$$

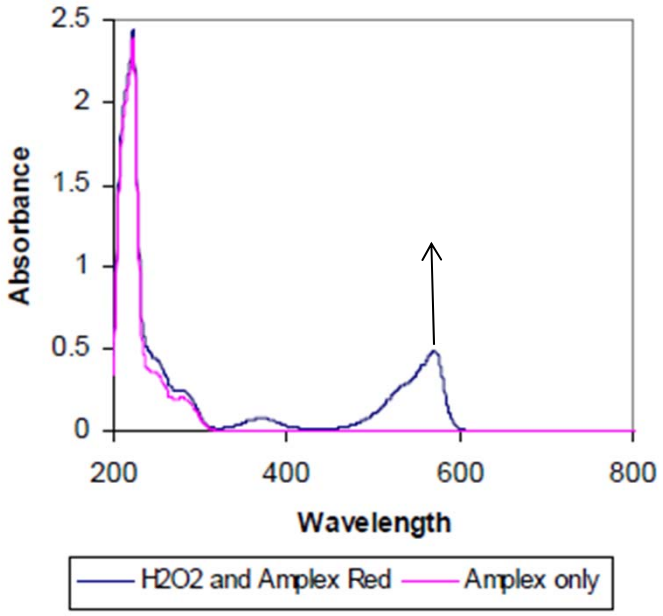
If $v_{B \rightarrow C} >> v_{A \rightarrow B}$, then $v_{A \rightarrow C} = v_{A \rightarrow B}$

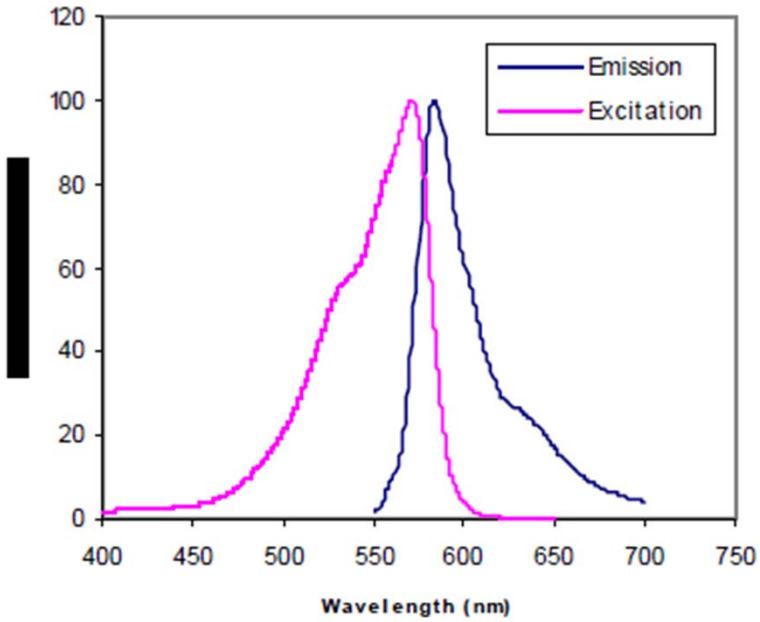
Depending on the products several indicator rxns can be used.



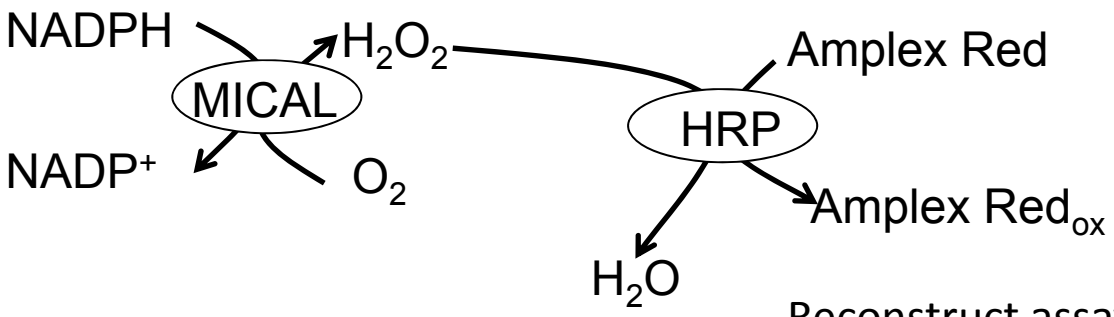
H_2O_2 production is often measured by coupling it to Horseradish Peroxidase in the presence of Amplex red by fluorescence.



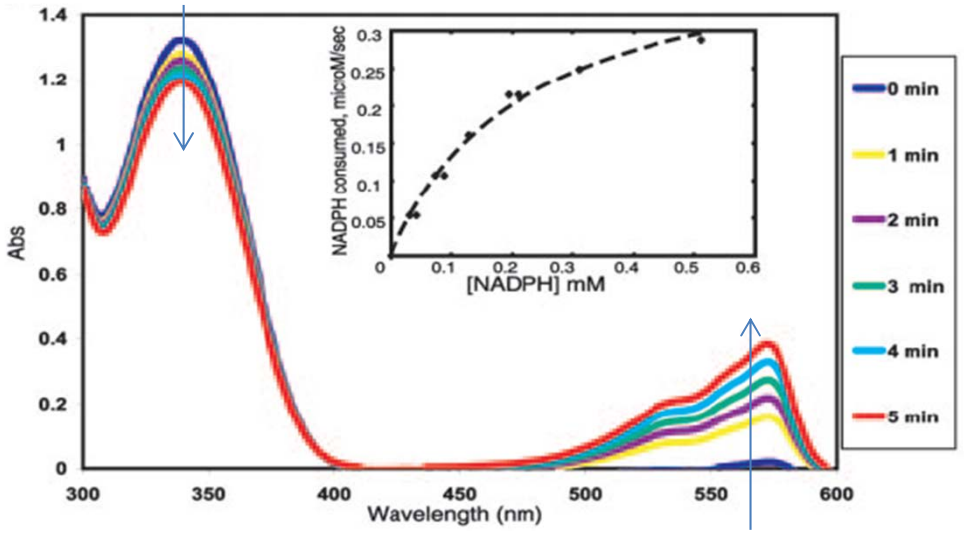




Possible artifacts: The HRP coupled assay of MICAL-MO

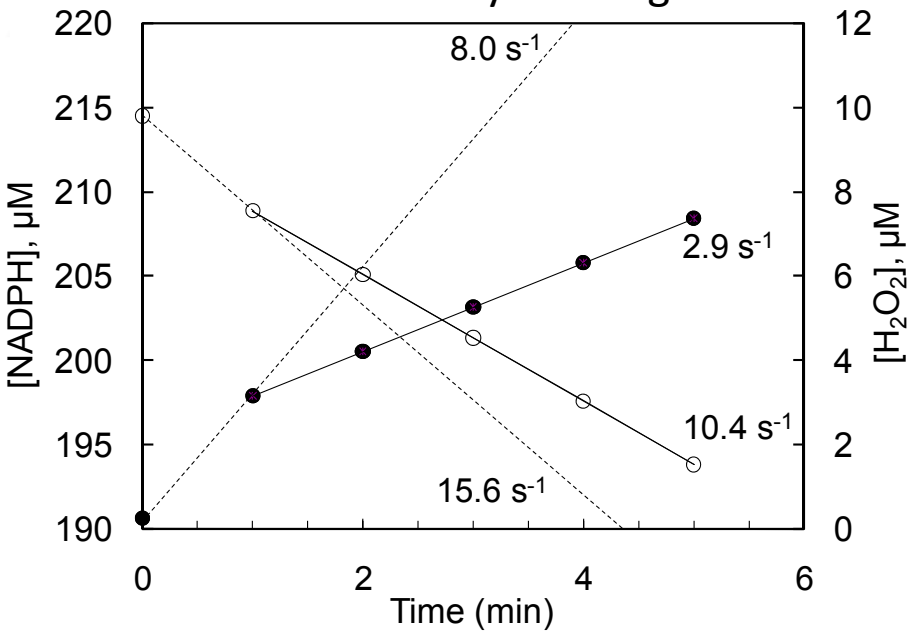


NADPH oxn



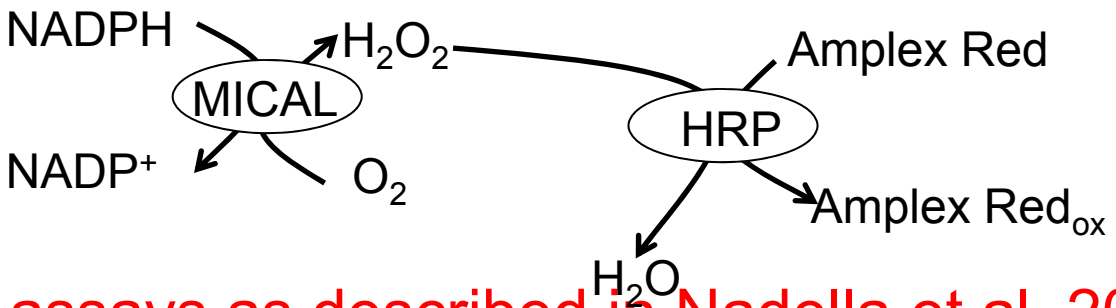
Amplex red oxn

Reconstruct assay from figure:

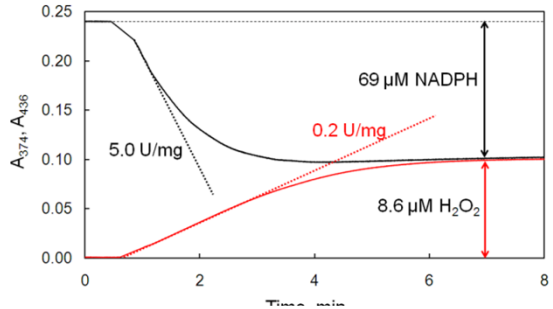


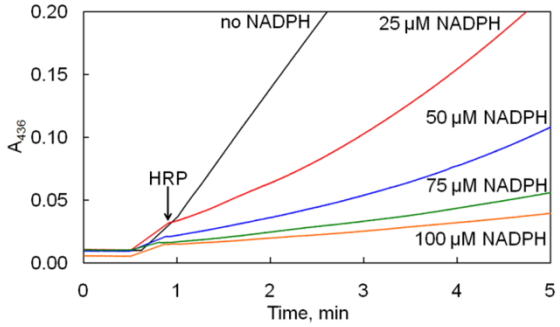
Turnover number: 15.6 or 10.4/s by monitoring NADPH oxidation vs 8 or 2.9/s by monitoring Amplex red oxidation

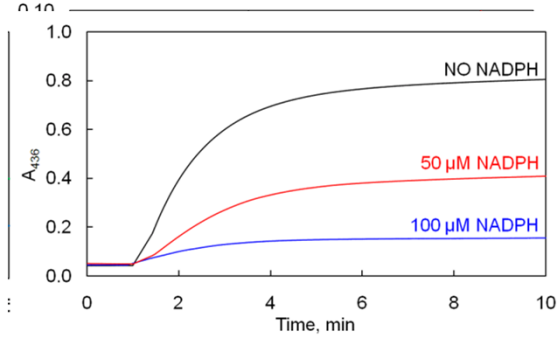
Figure 5, PNAS, 2005



Set up activity assays as described in Nadella et al. 2005 and find that:







- HRP, Amplex red and H_2O_2 enhance NADPH oxidation
- NADPH inhibits HRP
- NADPH lowers the amout of H2O2 detected at the end of the reaction

Cnclusions:

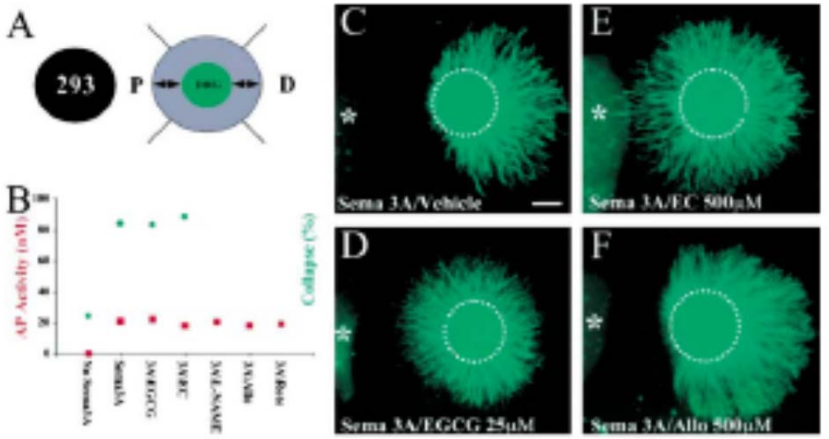
- the spectrophotometric coupled assay cannot be used to assay MICAL NADPH oxidase activity
- Rather just measure NADPH oxidation at 340 nm

MICAL controls axon growth in response to semaphorins binding to their Plexin receptor

Inhibition of MICAL removes axon growth inhibition

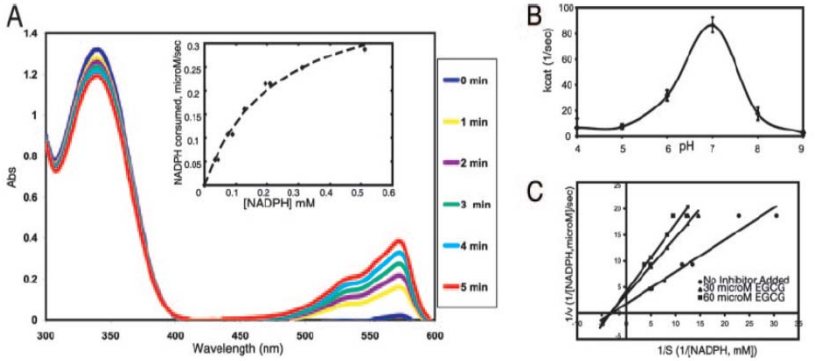
Inhibition of MICAL may promote nerve regeneration after spinal chrd injury

Terman et al., Cell 2001: Treatment of Dorsal Root Ganglion cells with the main green tea component (-) epigallocatechin gallate EGCG mimics MICAL LOF mutants by acting as a specific inhibitor of MICAL-MO function



PNAS, 2005:

EGCG is a specific and potent noncompetitive inhibitor of mMICAL-MO with Ki, 0.5 mM:



Conclusions: EGCG could be used as drug to promote axon regeneration

Effect (and structure) of (-) epigallocatechin gallate (EGCG) is very similar to the effect of xanthofulvin , a potent inhibitor of Sema3A, which has been shown to promote recovery form spinal cord injury in rats

A selective Sema3A inhibitor enhances regenerative responses and functional recovery of the injured spinal cord

Shinjiro Kaneko[1–3,9], Akio Iwanami[1,2,4,9], Masaya Nakamura[1], Akiyoshi Kishino[5], Kaoru Kikuchi[5], Shinsuke Shibata[2], Hirotaka J Okano[2], Takeshi Ikegami[1], Ayako Moriya[2], Osamu Konishi[5], Chikao Nakayama[5], Kazuo Kumagai[5], Toru Kimura[5], Yasufumi Sato[6], Yoshio Goshima[6], Masahiko Taniguchi[7], Mamoru Ito[8], Zhigang He[3], Yoshiaki Toyama[1] & Hideyuki Okano[2]

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In Vitro* and *in Vivo* Characterization of a Novel Semaphorin 3A Inhibitor, SM-216289 or Xanthofulvin

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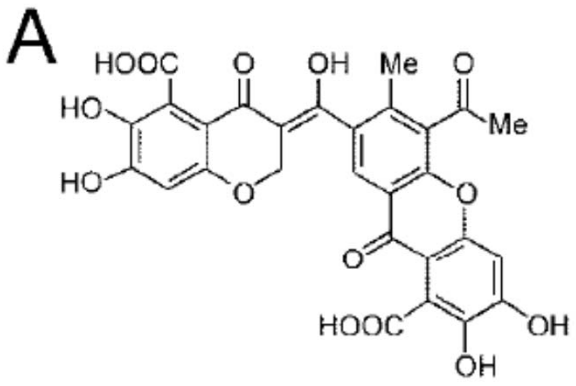
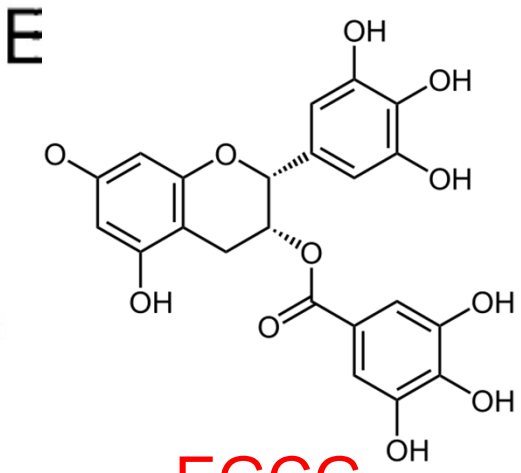


SM-216289



EGCG

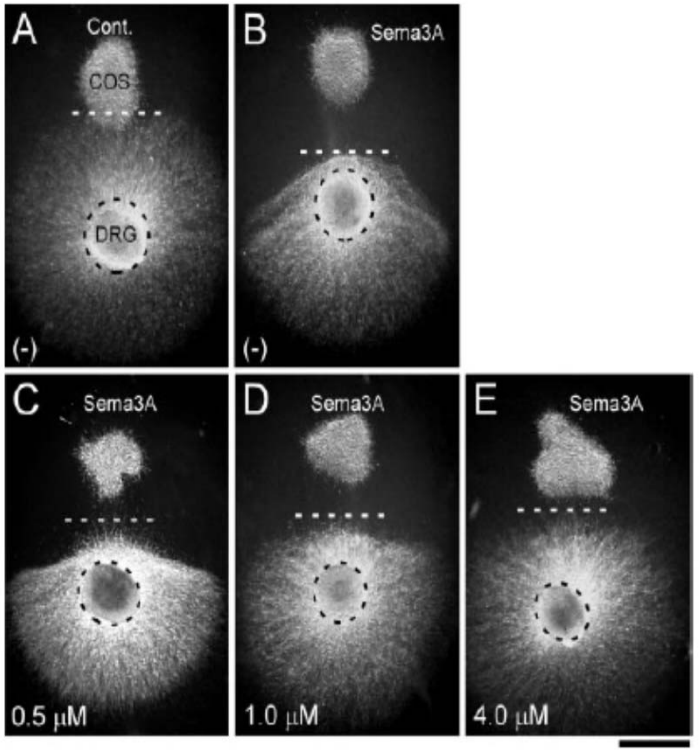
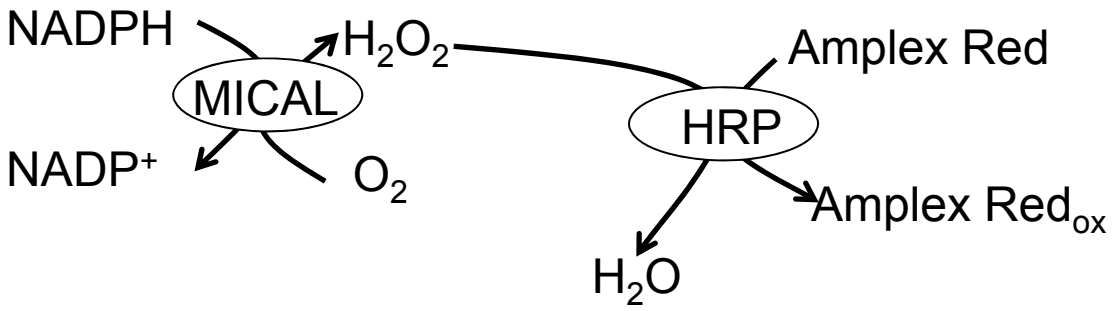
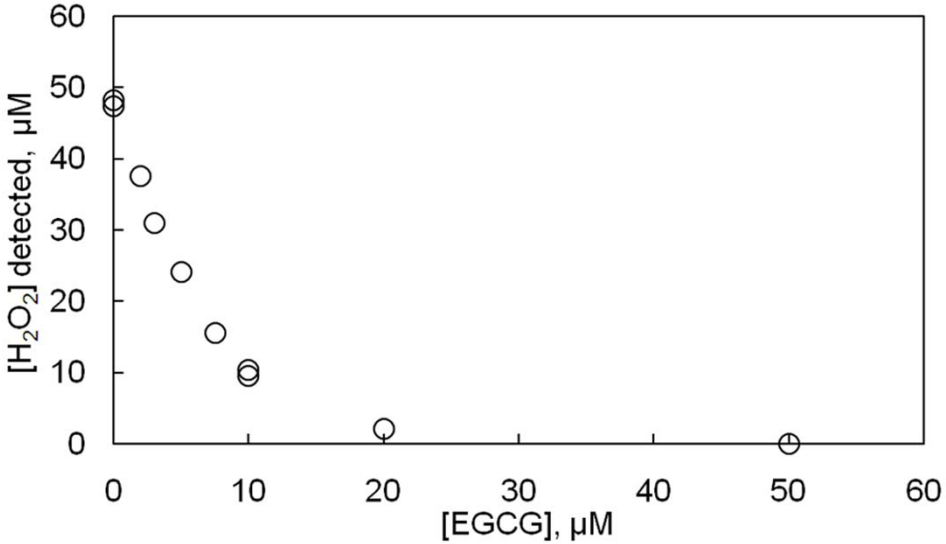


FIG. 2. **SM-216289 persistently inhibits the repulsive activity of Sema3A.** E8 chick DRG explants were co-cultured with control COS7 cell aggregate (*A*) or Sema3A-expressing COS7 cell aggregates (Sema3A-COS) (*B–D*) for 2 days in a collagen gel matrix in the presence or absence of SM-216289. The concentrations of SM-216289 were 0 μM (*A* and *B*), 0.5 μM (*C*), 1.0 μM (*D*), or 4.0 μM (*E*). *Dashed circle* indicates the location of explanted DRG. *Dashed line* indicates the position of neurite tips extending in the direction of COS7 cell aggregate. *Scale bar*, 0.5 mm.

The activity assay is critical to gather sound data

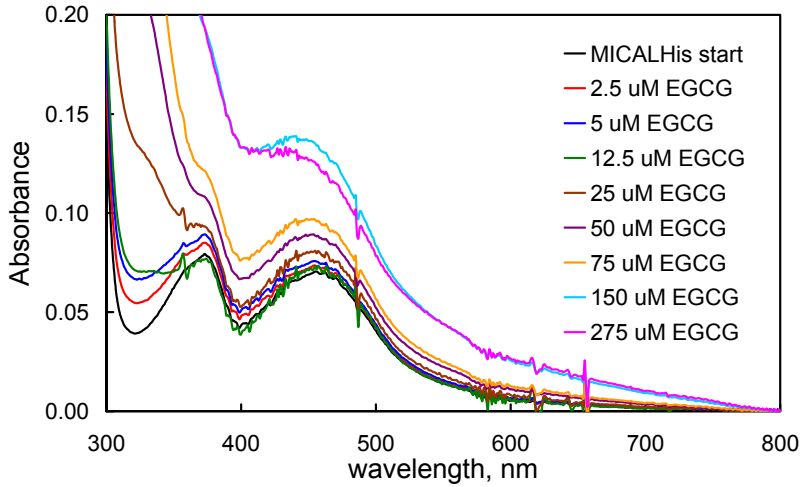


ECGC as a catecol scavenges H_2O_2

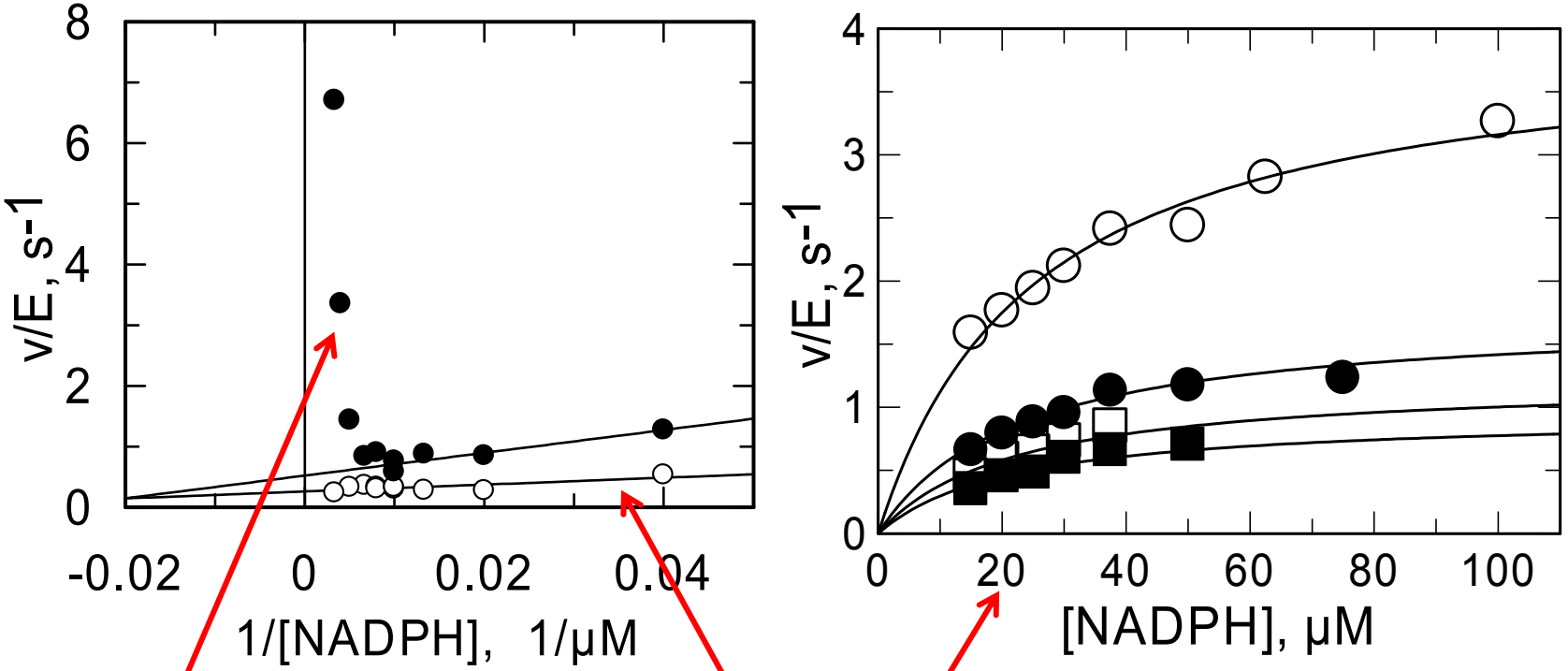


Quantitation of H_2O_2 (50 µM) with HRP/o-dianisidine in the presence of EGCG

EGCG causes MICAL denaturation as revealed by enzyme titration

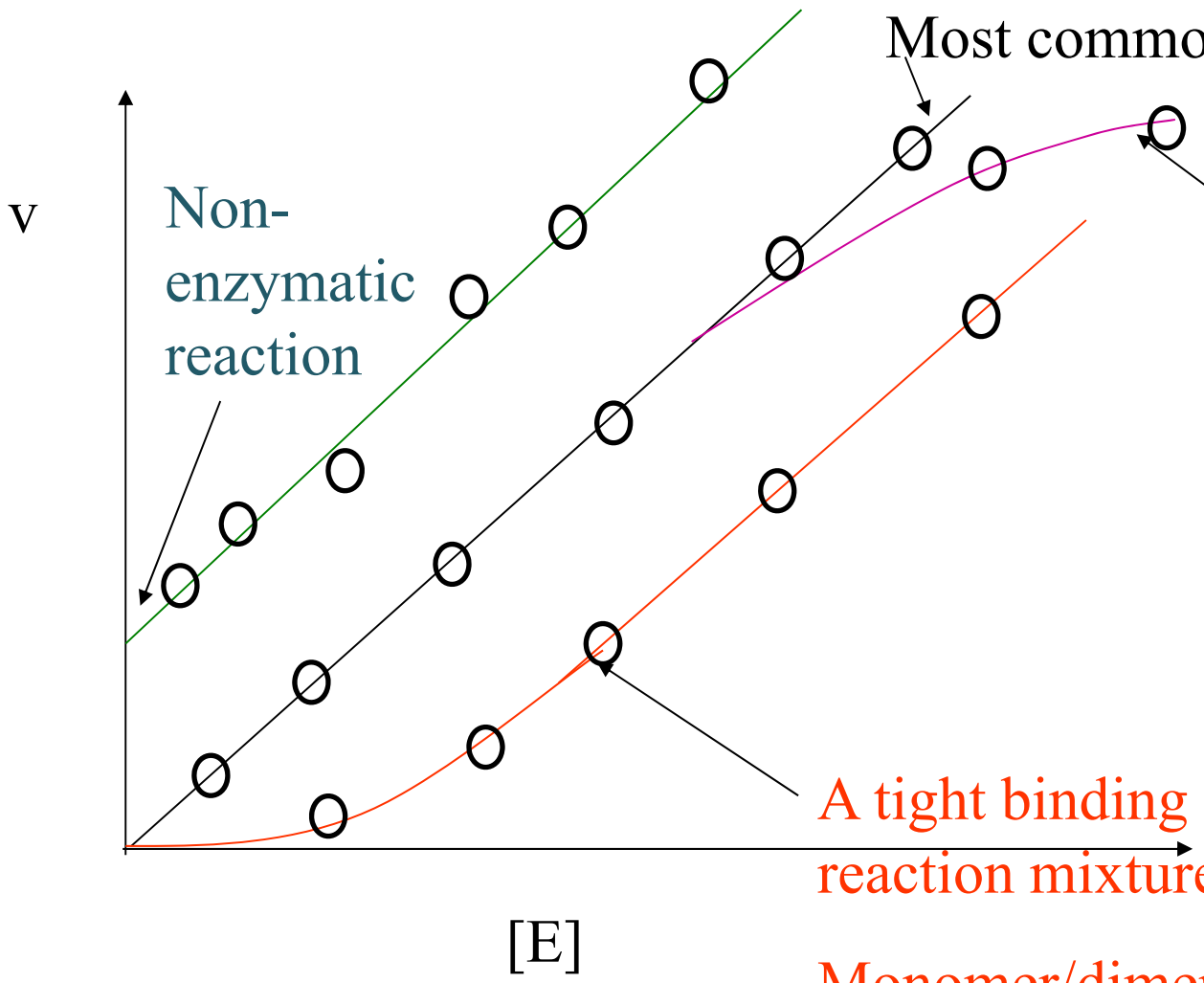


By monitoring NADPH oxidation at 340 nm (no HRP, no dye but with hMICAL), EGCG is a much less potent inhibitor than previously reported



- NonCompetitive inhibition but K_{is} = K_{ii} = 17 µM >> 0.5 µM
- Excess inhibition at high NADPH due to enzyme denaturation?

Dependence of v from $[E_T]$: deviations from linearity are informative



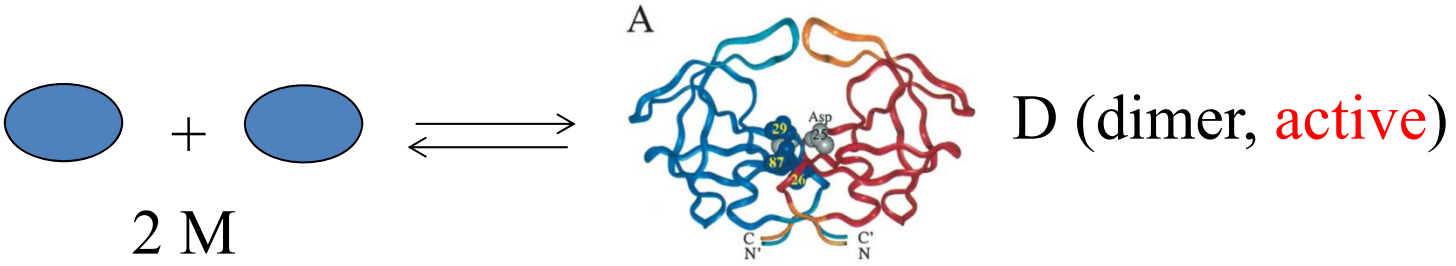
Reaction too fast to measure the initial velocity;

Monomer/dimer equilibrium; dimer is inactive

A tight binding inhibitor in the reaction mixture,

Monomer/dimer equilibrium and the monomer is inactive

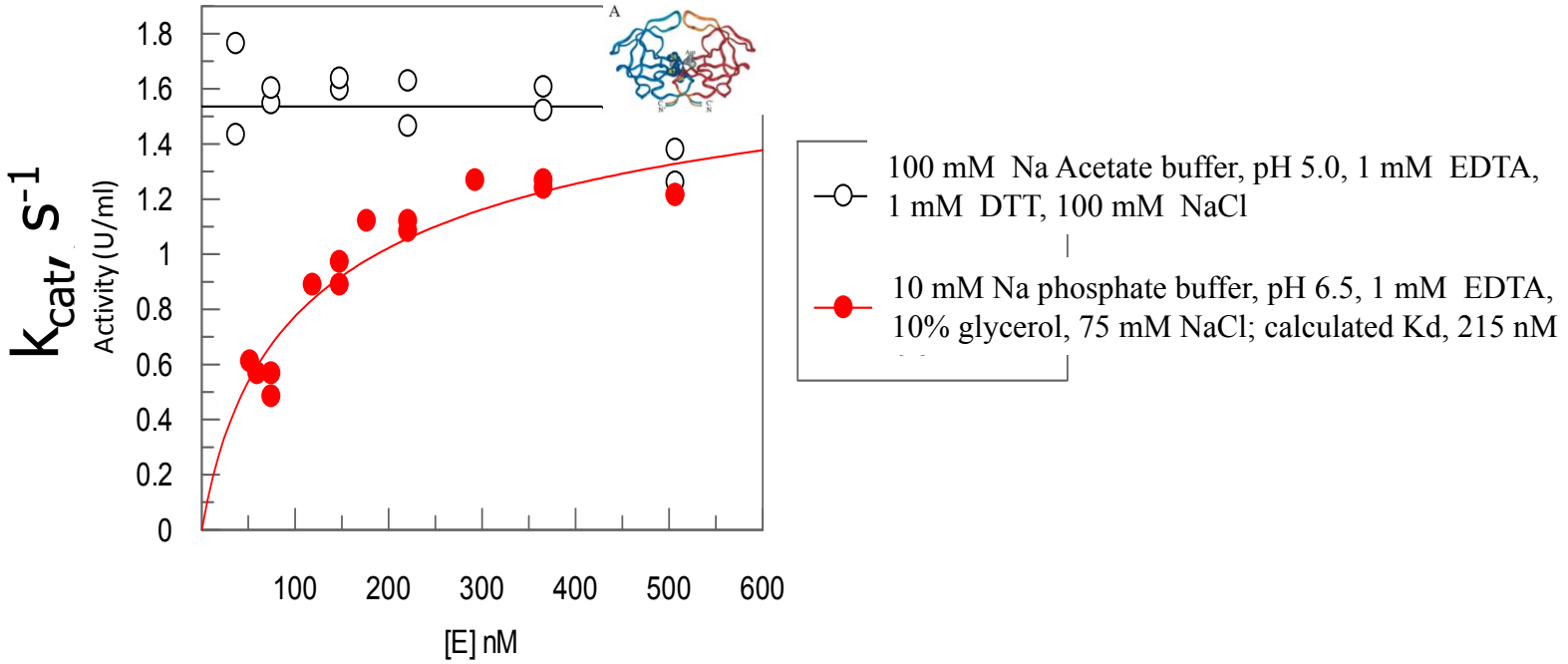
Activity assays allow to monitor the dissociation of the HIV-1 protease dimer.



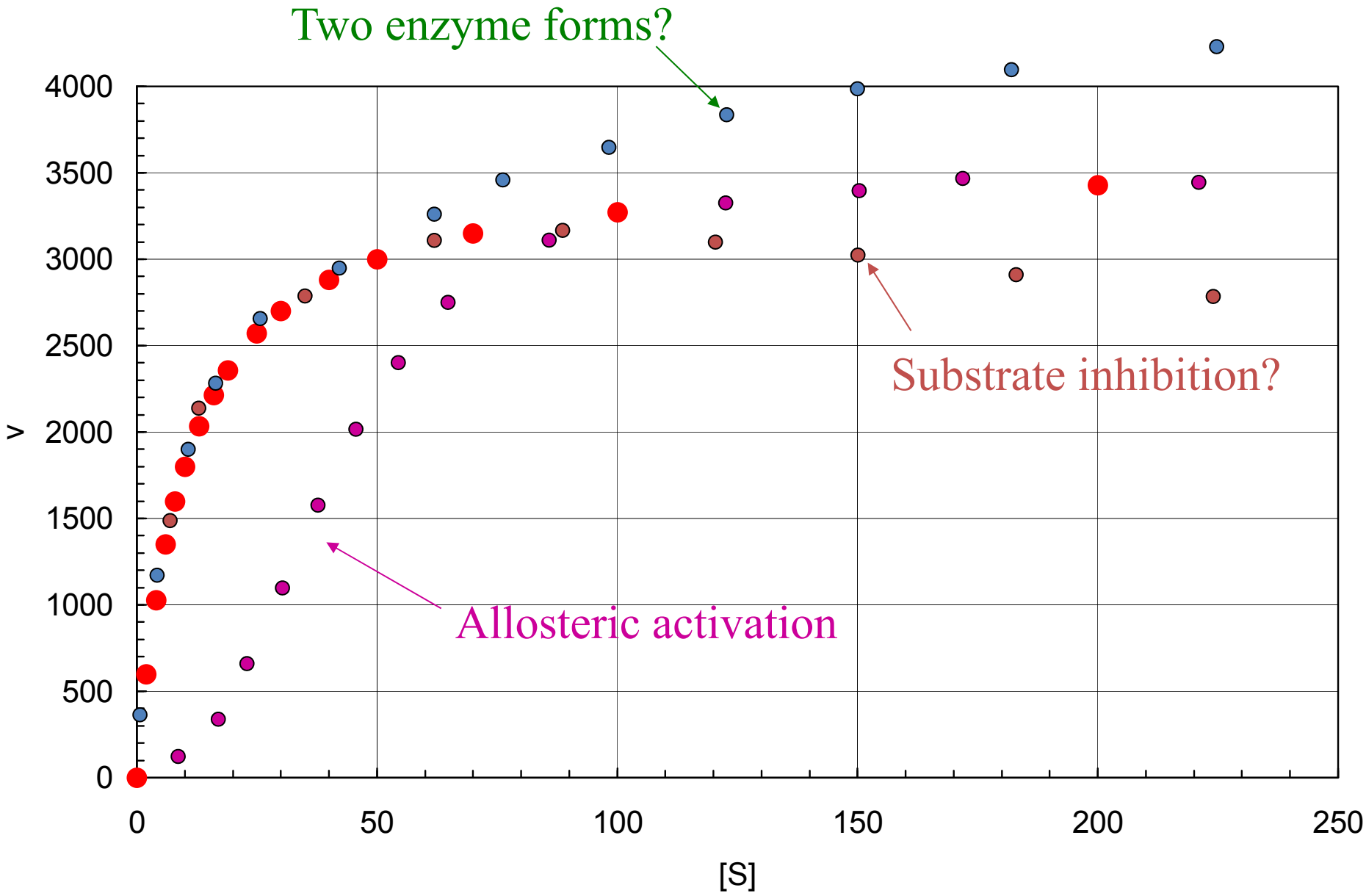
$v = k_{cat} * [Dimer]$

$$K_d = \frac{[M]^2}{[D]}$$

$$[E_{tot}] = 2*[D] + [M]$$



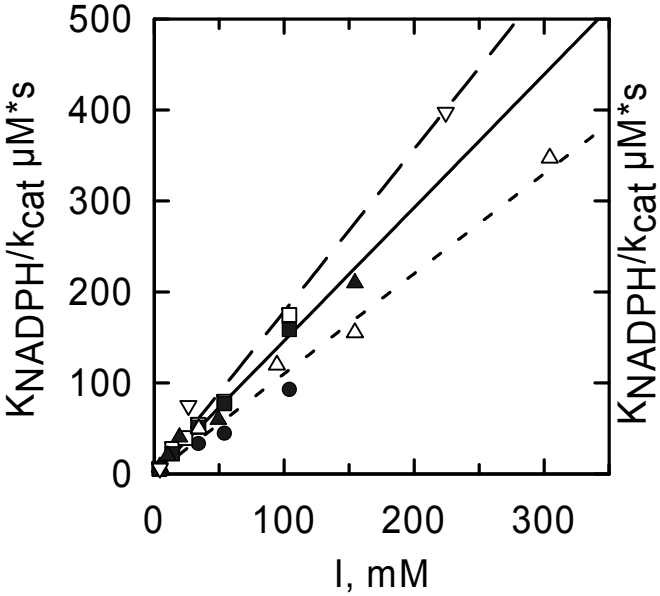
Deviations from the Michaelis-Menten equation



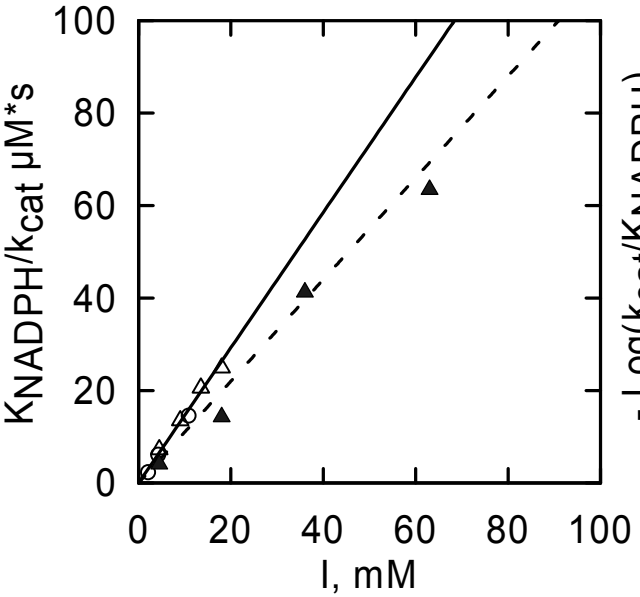
MICAL-MO NADPH oxidase reaction is sensitive ionic strength and the type of anions:

Strong effect on V/K_{NADPH} mainly due to effect on Km due to:

Competition between anions and NADPH

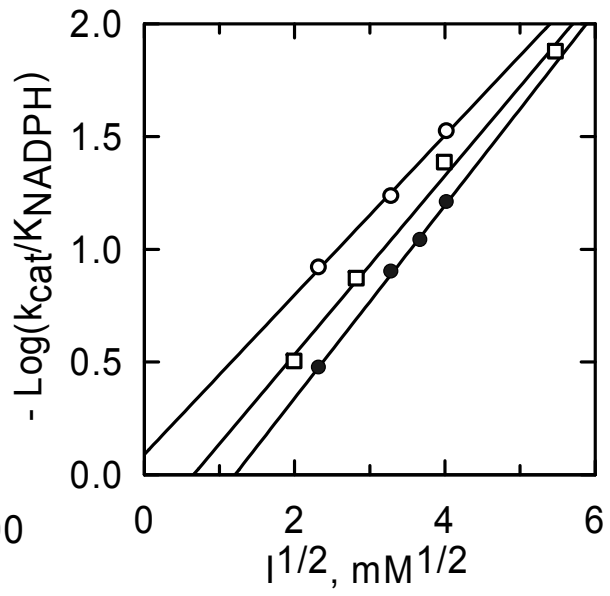


Buffer :20 mM Hepes/NaOH, pH 7.0:
●, Na acetate; △, Mg acetate;
□, NaCl; ■, KCl; ▲, KCl_2;
▽, sodium phosphate.



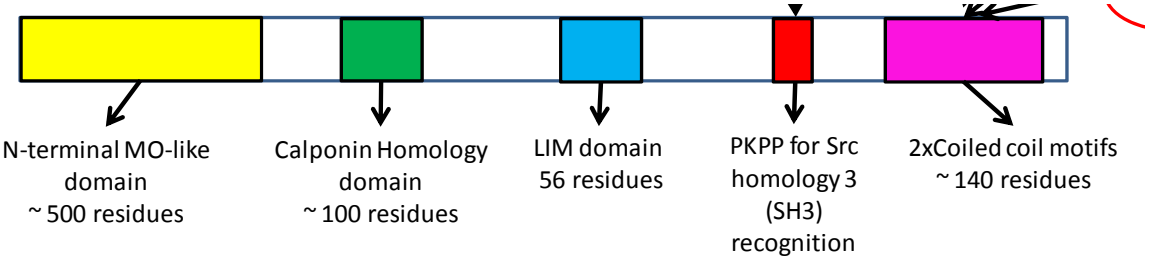
Buffer:
Hepes/NaOH buffer (○),
Tris-chloride (△)
Tris-acetate (▲).

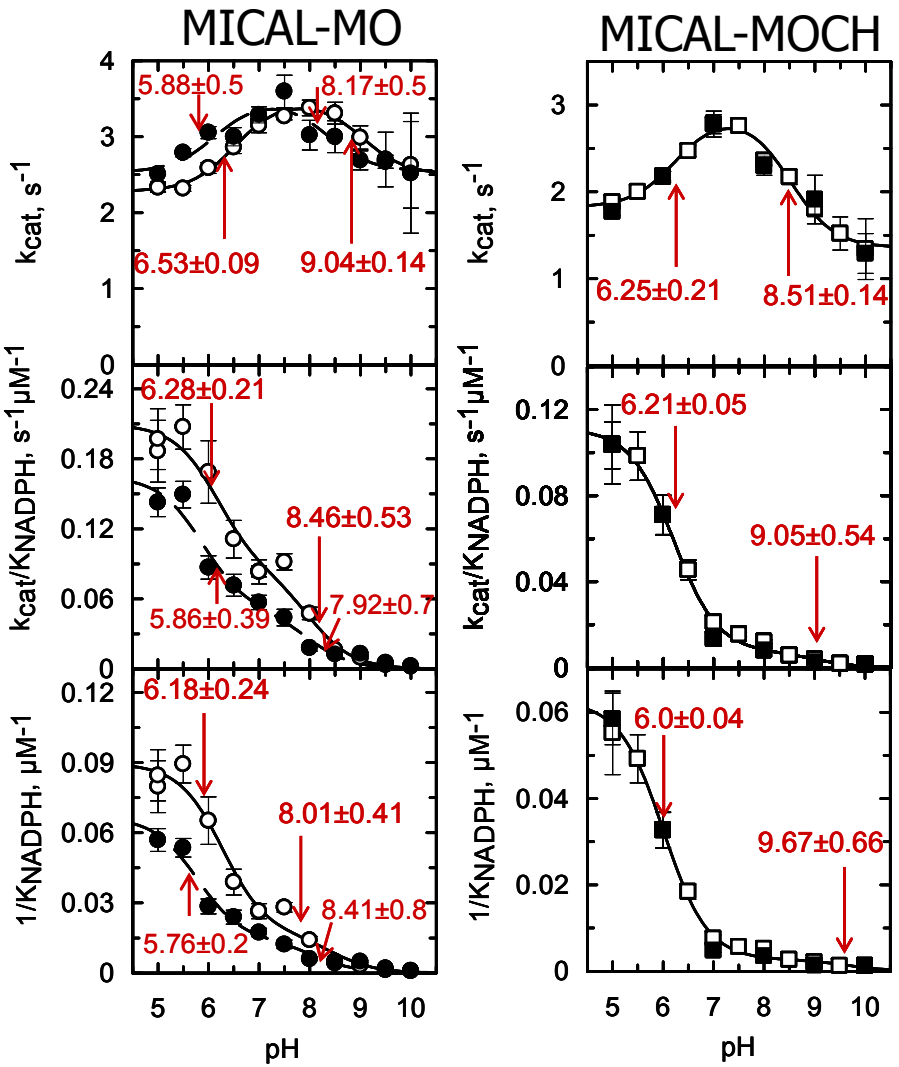
Electrostatic effects



Buffer;
imidazol-chloride (○),
imidazol-acetate (□),
Bis-Tris-acetate (●).
Fit to Debye-Huckel equation

Design mixed buffer for pH studies to minimize ions and I effects





Effect of the pH on the NADPH oxidase activity of MICAL-MO and –MOCH at constant (low) ionic strength

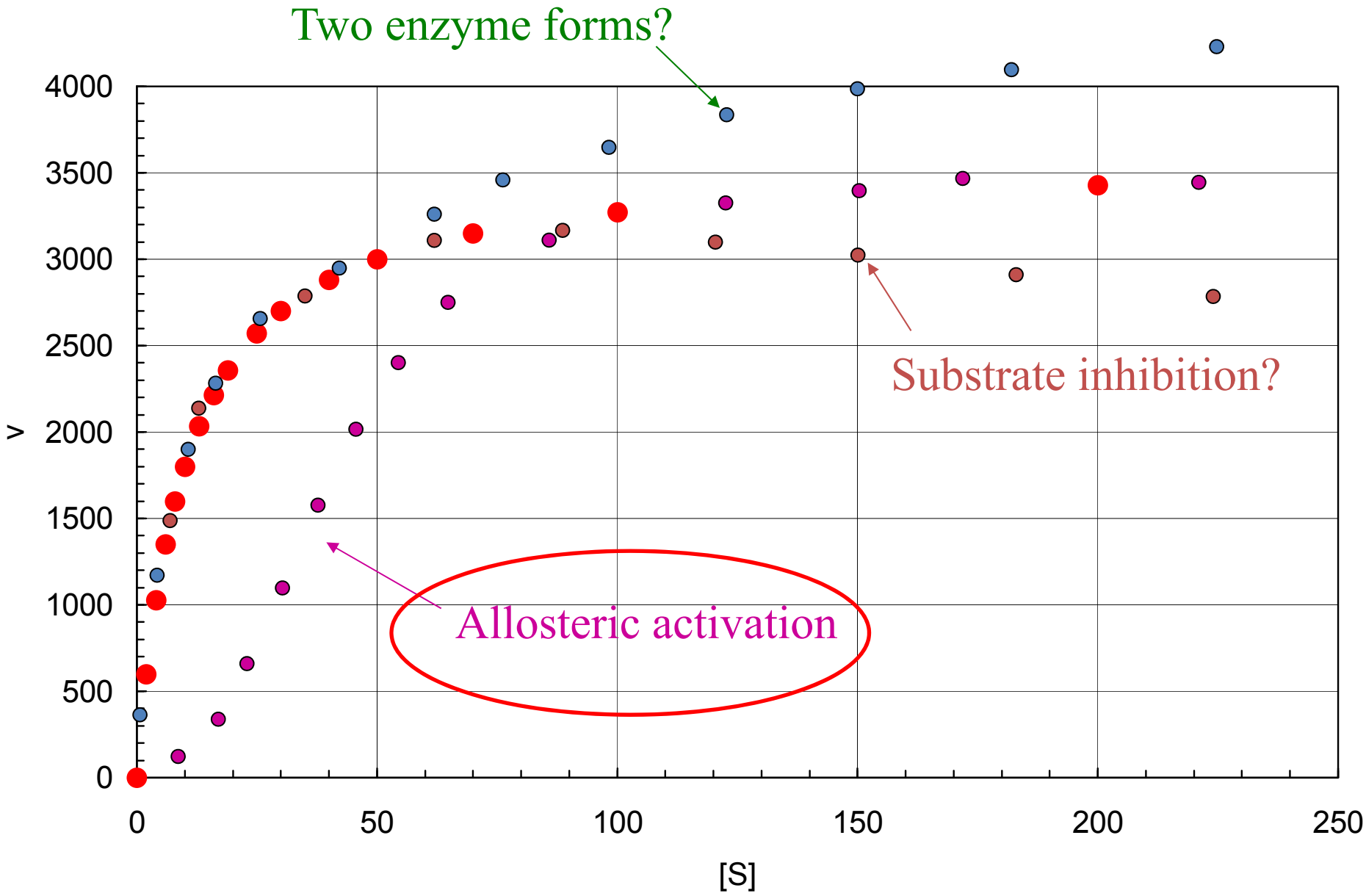
k_{cat} varies only 2-3 fold; limits at low and high pH are >0

Km increases dramatically as the pH increases

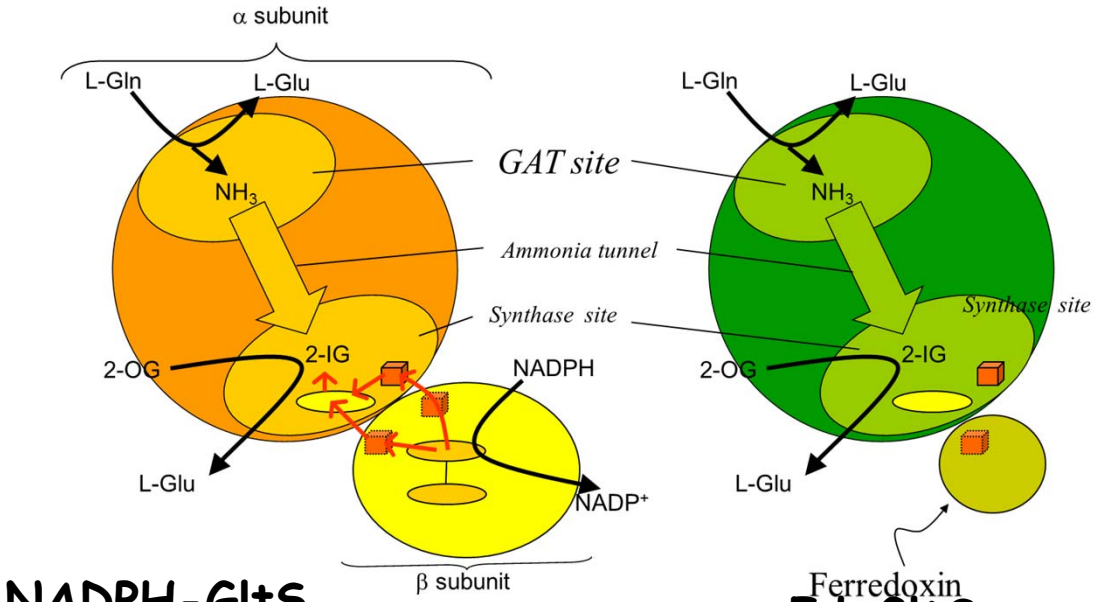
We run assays at pH 7: a compromise also for NADPH stability

Mixed buffers with constant ionic strength (□,○: acetate/imidazole/Tris, I = 10 mM; ■,●: formate/imidazole/Tris, I = 12.5 mM)

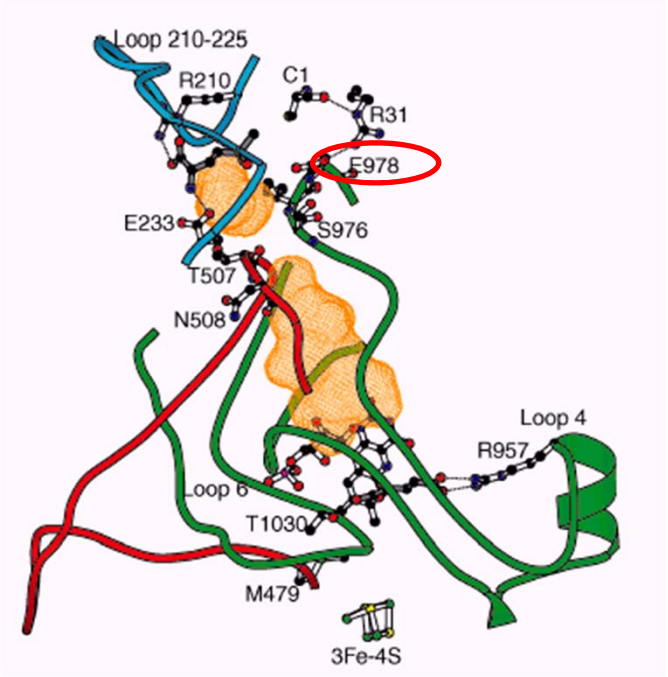
Deviations from the Michaelis-Menten equation



Glutamate synthase is complex Fe/S flavoprotein , essential for ammonia assimilation



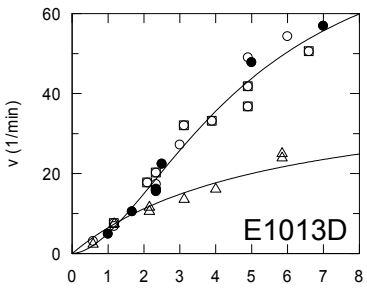
NADPH-GltS **Fd-GltS**



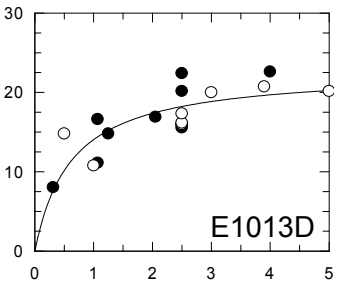
As an amidotransferase the glutaminase site is connected to the synthase site by an intramolecular tunnel, and the glutaminase site is activated only when 2-OG is bound to the synthase site and the cofactors are properly reduced.

E978 (E1013 in FdGltS) is well positioned to signal the presence of 2-OG to the glutaminase and to the tunnel entrance: E-to-D/N/A substitutions

Vary L-Gln @ 2.5 mM 2OG



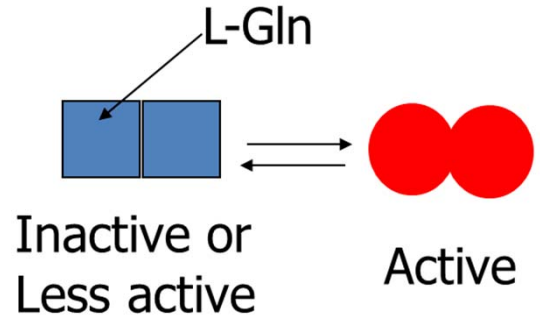
Vary 2OG @ 2.5 mM L-Gln



E1013D/FdGltS:

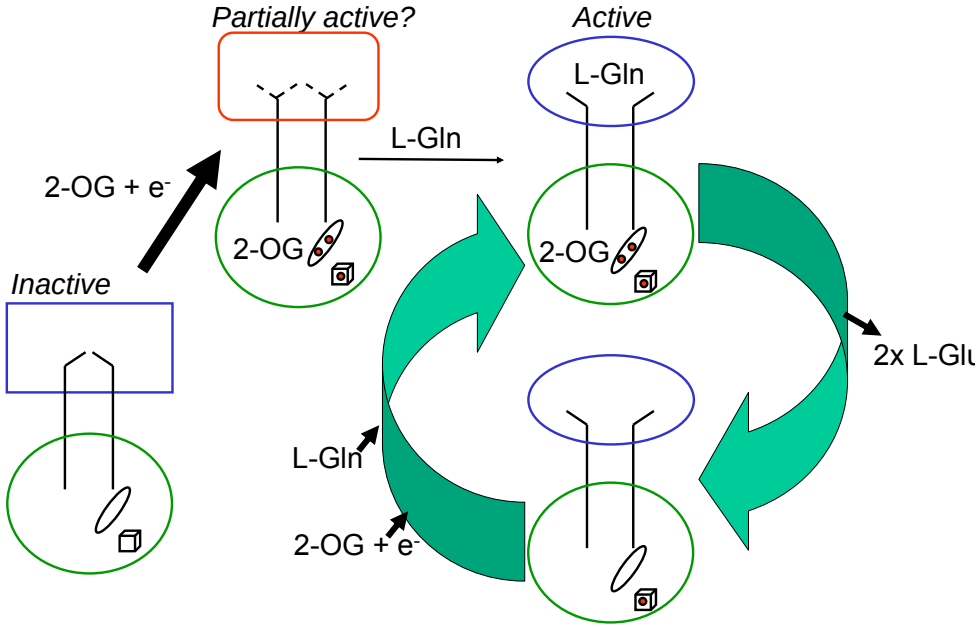
Coupled but sigmoid kinetics when L-Gln is varied at fixed (high) 2-OG

Sigmoid kinetics cannot be explained with a "classical" allosteric effect because SAXS told us that FdGltS is monomeric.



Data are consistent with one of the several Schemes leading to sigmoid kinetics in monomeric enzymes: two-step activation process

(Segel, Enzyme kinetics, 1978, Wiley)



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GUEST COMMENTARY

Ten Commandments: Lessons from the Enzymology of DNA Replication

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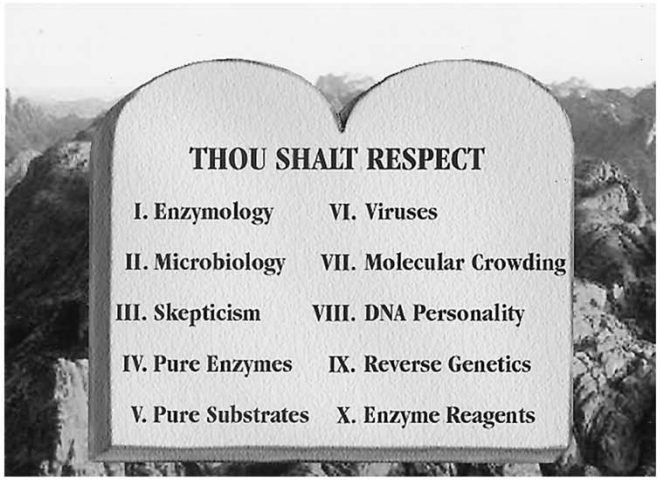

FIG. 4. Ten commandments. Lessons from the enzymology of DNA replication.

I. RELY ON ENZYMOLOGY TO CLARIFY BIOLOGIC QUESTIONS

II. TRUST THE UNIVERSALITY OF BIOCHEMISTRY AND THE POWER OF MICROBIOLOGY

III. DO NOT BELIEVE SOMETHING BECAUSE YOU CAN EXPLAIN IT

IV. DO NOT WASTE CLEAN THINKING ON DIRTY ENZYMES

By Efraim Racker

V. DO NOT WASTE CLEAN ENZYMES ON DIRTY SUBSTRATES

Protein overproduction in heterologous host: some useful references

Microbial Cell Factories

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Review

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Soluble expression of recombinant proteins in the cytoplasm of *Escherichia coli*

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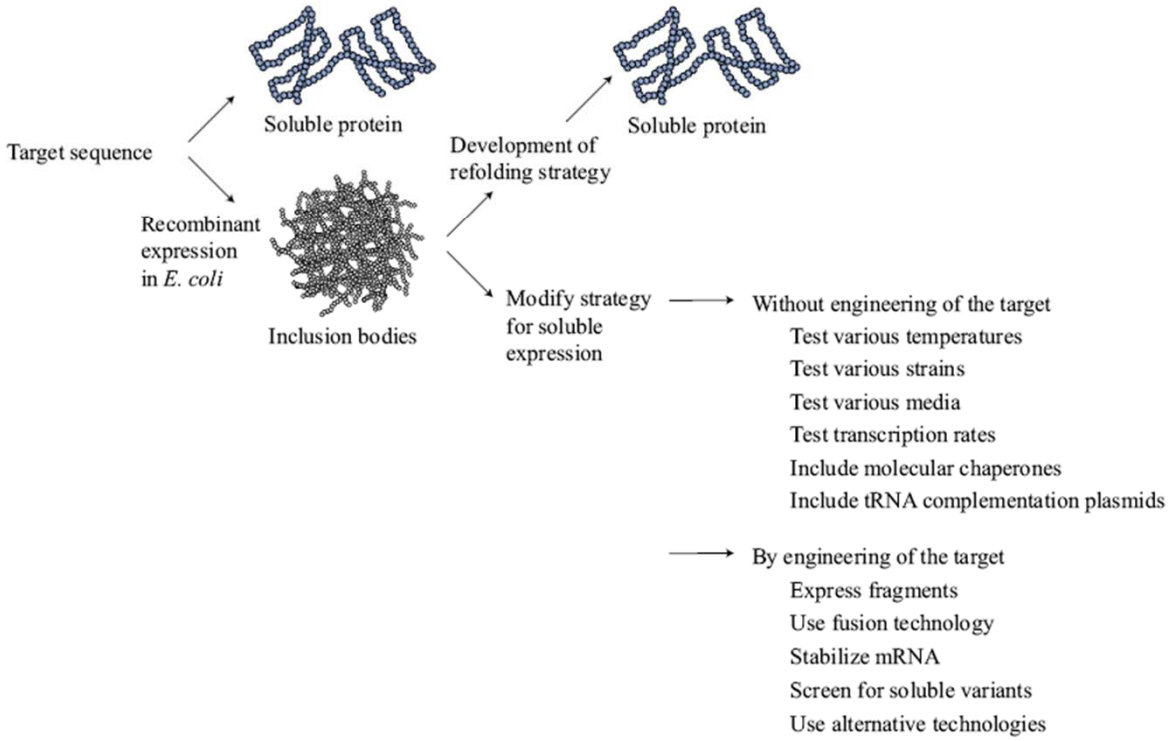

Figure 1
Downstream applications employed to obtain soluble proteins from recombinant *E. coli*. As a common trait the *in vivo* strategies aims at lowering the metabolic burden associated with recombinant expression. Some of the mentioned strategies have therefore merely indirect influence on folding such as the use of tRNA complementation plasmids and stabilization of mRNA (see text and ref [1] for details).

Structural biology consortia; Specialized laboratories

Protein production and purification

Structural Genomics Consortium[1–3], Architecture et Fonction des Macromolécules Biologiques[4], Berkeley Structural Genomics Center[5], China Structural Genomics Consortium[6,7], Integrated Center for Structure and Function Innovation[8], Israel Structural Proteomics Center[9], Joint Center for Structural Genomics[10,11], Midwest Center for Structural Genomics[12], New York Structural GenomiX Research Center for Structural Genomics[13–17], Northeast Structural Genomics Consortium[18,19], Oxford Protein Production Facility[20], Protein Sample Production Facility, Max Delbrück Center for Molecular Medicine[21], RIKEN Structural Genomics/ Proteomics Initiative[22] & SPINE2-Complexes[23,25]

Nature methods (2008) 5, 135

Table 2 | Summary of approaches used by SG centers

Center	Main target sources	Cloning method	Expression promoter	Affinity tags	Small-scale expression method	Scale-up cultivation method	Purification strategy	Protein characterization	References
Structural Genomics Consortium	Human and human pathogens	LIC	T7	6His	96-well plates	1–2 l in Tunair shake flasks	IMAC and gel filtration using ÄKTA systems	ESI-MS	16,19
Architecture et Fonction des Macromolécules Biologiques	Mammalian viruses, higher eukaryotes, bacteria, phages	Gateway	T7	6His	96-well plates	1–5 l in shake flasks	IMAC and gel filtration using ÄKTA systems	MALDI-TOF	32,51
Berkeley Structural Genomics Center	Bacteria	LIC	T7	6His	96-well plates	1 l cultures in Fembach flasks	IMAC and gel filtration using ÄKTA systems	DLS, MALDI, ANSEC	85
China Structural Genomics Consortium	Human	LIC, restriction enzyme–based	T7	6His	3 ml culture in test tubes	2 l in Erlenmeyer flasks	IMAC, and ion exchange chromatography and gel filtration	SDS-PAGE, DLS and mass spectrometry	8,9
Integrated Center for Structure and Function Innovation	*Mycobacterium tuberculosis*, *Bacillus subtilis*, *Thermotoga maritima*	Restriction enzymes and LIC	T7 and arabinose	6His	96-well plates	0.5–1 l in soda bottles or baffled shake flasks	IMAC and gel filtration	SDS-PAGE, densitometry, DLS and MALDI	

Table 2 | (continued)

Center	Main target sources	Cloning method	Expression promoter	Affinity tags	Small-scale expression method	Scale-up cultivation method	Purification strategy	Protein characterization	References
Israel Structural Proteomics Center	Higher eukaryotes, human pathogens	Restriction enzyme–based or LIC	T7	N-6His	4 ml culture in test tubes	0.5 l culture in 2-l shake flasks or 1.25 l culture in 5-l flasks (total 5–6 l per large-scale production)	IMAC, gel filtration, ion exchange chromatography and TEV cleavage	MALDI and ESI-MS	10
Joint Center for Structural Genomics	Bacteria	Polymerase incomplete primer extension	Arabinose	6His	96-well plates, ANSEC and mass spectromerty	Parallel 12–96 cultures in GNFermentor	IMAC, TEV cleavage, IMAC subtraction, ion exchange chromatography and gel filtration if necessary	ANSEC, LC-MS, SDS-PAGE	18,86–88
Midwest Center for Structural Genomics	Bacteria	LIC	T7	6His	96-well plates	Plastic bottles	IMAC, TEV cleavage, gel filtration	SDS-PAGE	89
New York Structural GenomiX Research Center for Structural Genomics	Human and >130 other species (ATCC and gene synthesis)	Topo (blunt)		C-6His and N-6His-Smt3	96-well plates	1–3 l shake flasks SeMet high yield	ÄKTAxpress and Ni-NTA column purification and gel filtration	MALDI and ESI-MS; protein identification by mass spectrometry and/or DNA sequencing	1
Northeast Structural Genomics Consortium	Prokaryotes and eukaryotes, including human	LIC	T7	6His	96-well plates	1–2 l in baffled shake flasks	IMAC, gel filtration using ÄKTAxpress, ion exchange chromatography if required	Caliper microfluidics, MALDI-TOF mass spectrometry, light scattering, NMR	13
Oxford Protein Production Group (SPINE)	Bacteria, human, viral pathogens	LIC	T7; β-actin or hCMV for mammalian cells	N- or C-6His	96-well plates; 25-cm^2 dishes for mammalian cells	1–2 l cultures	IMAC and gel filtration using ÄKTA systems	SDS-PAGE, ESI-MS, MALDI-TOF MS; LC-ESI-MS followed by ZIC-HILIC for glycosylated proteins	5,14,51,90
Protein Sample Production Facility, Max Delbrück Center for Molecular Medicine	Human and higher eukaryotes	Restriction enzyme–based, Gateway	T5 and T7	Mainly N-7His, occasionally N-GST or N-MBP	1–10 ml culture	1–8 l in shake flasks	IMAC and TEV cleavage, IMAC and gel filtration, and ion exchange chromatography using ÄKTA systems if necessary	Mass spectrometry, DLS	2,3,7
RIKEN Structural Genomics/ Proteomics Initiative	Human, mouse, bacteria, and archaea	Two-step PCR and TA cloning	T7	Histidine affinity tag (HAT)	30 µl in cell-free synthesis in 96-well plates	9–27 ml dialysis cell-free synthesis	IMAC and TEV cleavage, IMAC subtraction, ion exchange chromatography, gel filtration using ÄKTA systems if necessary	DLS, NMR, MALDI-TOF and quadrupole-TOF tandem mass spectrometry	72–76
SPINE2-Complexes	Human, viral proteins involved in subversion of human signaling pathways	LIC	T7; β-actin or hCMV for mammalian cells	N- or C-6His tag	96-well plates; 25-cm^2 dishes for mammalian cells	1–2 l cultures	IMAC and gel filtration using ÄKTA systems	SDS-PAGE, ESI-MS, MALDI-TOF mass spectrometry; LC–ESI-MS followed by ZIC-HILIC for glycosylated proteins	51,71

ESI-MS, electrospray ionization–mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization–time of flight; DLS, dynamic light scattering; ANSEC, analytical size-exclusion chromatography; ZIC-HILIC, zwitterionic chromatography-hydrophilic interaction liquid chromatography; LC-MS, liquid chromatography–mass spectrometry; SeMet, selenomethionine.

to express the full-length protein to address a particular biological step size between the

BOX 1 SUMMARY OF CONSENSUS PROTOCOL

- Obtain the cDNA by amplifying either genomic DNA (prokaryotic genes, or eukaryotic genes with no introns) or full-length, sequence-verified cDNAs (eukaryotes) or by total gene synthesis.
- Use ligation-independent cloning (LIC) to clone the full-length cDNA (or the fragment of interest) into an *E. coli* expression vector.
- Use T7 RNA polymerase–driven expression and an N-terminal oligohistidine tag (include a cleavage site for a sequence-specific protease to enable removal of the tag).
- Express the protein in a derivative of the *E. coli* BL21(DE3) strain, with induction at low temperature (15–25 °C) in rich medium and with good aeration. If expressing proteins from organisms that have codon biases differing from those used by *E. coli*, use a strain supplemented with the appropriate tRNA genes.
- Solubilize and purify the protein in a well-buffered solution containing an ionic strength equivalent to 300–500 mM of a monovalent salt, such as NaCl.
- Use immobilized metal affinity chromatography (IMAC) as the initial purification step.
- If additional purification is required, use size-exclusion chromatography (gel filtration). If necessary, use ion exchange chromatography as a final 'polishing' step.
- The affinity tag may be removed to minimize non-native sequences in the recombinant protein and to achieve further purification. Use a recombinant, hexahistidine-tagged protease and reapply the sample to IMAC column to remove the protease and any cellular proteins that bound to the metal affinity resin.

Table 1

Comparison of expression systems used for biopharmaceutical production [4,6,7]

	Higher eukaryotes		Yeast		*Escherichia coli*
Ease of genetic modifications	Moderate		Simple		Simple
Cultivation	Slow growth rates, expensive complex (or synthetic) media required		Fast and robust growth, defined minimal media		Fastest growth, defined minimal media
Contaminations	Risk of viral contaminations, viral clearance required		Little risks of endotoxins or viral DNAs		Endotoxins presence requires thorough purification, possible phage infections
Post translational modifications (PTMs)	Closely resembling human PTMs; usually mixtures of several glycoform variants		Most human PTMs achievable, but natural glycosylation patterns differ from humans, hypermannosylation, engineered strains can achieve human glycoforms and high uniformity		Limited set of PTMs, some human PTMs (e.g. glycosylation) difficult to achieve
Protein yields and secretory capacities	High yields, highly efficient secretion, high specific productivity		High yields, secretory capacities depending on the species		High expression capacities, secretion mostly inefficient, extensive purification and downstream processing required
Most commonly used species	Mammalian cells	Insect cells	*Pichia pastoris*	*Saccharomyces cerevisiae*	
Recently approved biopharmaceuticals[a]	32	2	2[b]	4	17
Additional information and specific differences between host species of the same class	Commonly used cell lines: CHO (Chinese Hamster Ovary), BHK (baby hamster kidney), murine-myeloma-derived NS0, SP2/0 cell lines [2] and HEK293	Baculo virus based systems most commonly used for transfection Easy scale up Contaminations less problematic Mammalianized glycosylation [5]	Efficient and selective secretion, often higher protein titers than *S. cerevisiae*, for example, [8**] Crabtree negative, high cell density cultivations GRAS status Hypermannosylation is less pronounced in *P. pastoris* and critical terminal α-1,3-mannose linkages were not observed [19], engineered strains providing fully humanized glycosylation not available for *S. cerevisiae*	Important eukaryotic model organism, high molecular- and cell biological knowledge Crabtree positive, leading to ethanol production	Fastest efficient expression system Inexpensive Well established processes suitable for mass production Folding problems may lead to the formation of inclusion bodies and require expensive refolding (yet, inclusion bodies provide a valuable strategy to achieve high protein yields and simple purification) Inefficient acetate metabolism may hamper high cell density cultivation of some strains

[a] Data from Walsh [1], time period: January 2006–June 2010, in total 58 biopharmaceuticals have been approved, two biopharmaceuticals produced in transgenic animals were not listed.
[b] In this number Jetrea by ThromboGenics is included (approved in 2012 and not listed by Walsh [1]).

Detergent binding explains anomalous SDS-PAGE migration of membrane proteins

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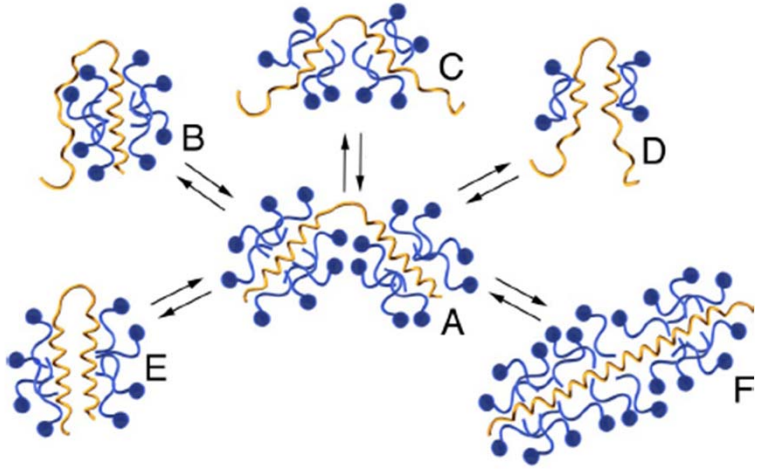

Fig. 5. Interrelationship between hairpin conformation and detergen binding. Hairpins (*yellow*) loaded with SDS molecules (*blue*) are represented The number of SDS molecules on each hairpin, and the number of turns o helical structure, are intended to illustrate relationships between relative levels of detergent binding and/or helicity in a quantitative manner. The necklace and bead structure typical of an unfolded membrane protein with it TM segments fully coated with detergent acyl chains (*A*) is shown at the center Potential alterations in SDS loading accompanying hydropathy reduction (*B–D*) are shown at the top; in some instances, regions with reduced hydrop athy may no longer intercalate with lipid acyl chains but instead may partitio closer to the micelle surface (*B*) or remain uncoated (*C* and *D*). In cases where detergent-TM domain interactions remain constant, conformational change may also alter SDS loading stoichiometry (*E* and *F*). Interconversions among al types of hairpin-detergent complexes are possible. See text for furthe discussion.

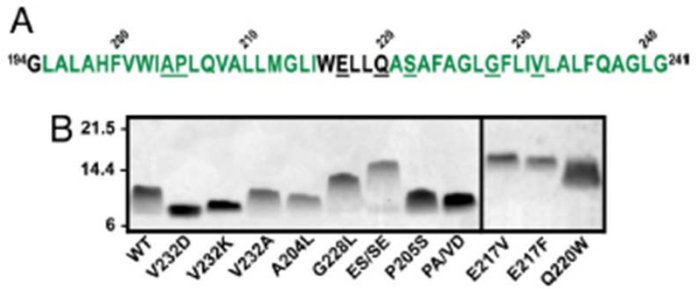

Fig. 1. Hairpin sequences and SDS-PAGE analysis. (*A*) Amino acid sequence of the WT TM3/4 hairpin. Residues predicted to be in helical (*green text*) or loop (*black text*) regions of CFTR are shown (34). Residues substituted in this work are underlined. (*B*) Representative SDS-PAGE of helical hairpin mutants. Positions of MW standards (in kDa) are indicated. This panel is a composite of two gels, as indicated by the solid line between lanes. PA/VD and ES/SE denote the P205A/V232D and E217S/S222E hairpins, respectively.

Abnormal SDS-PAGE migration of cytosolic proteins can identify domains and mechanisms that control surfactant binding

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http://onlinelibrary.wiley.com/doi/10.1002/pro.2107/full#fig6

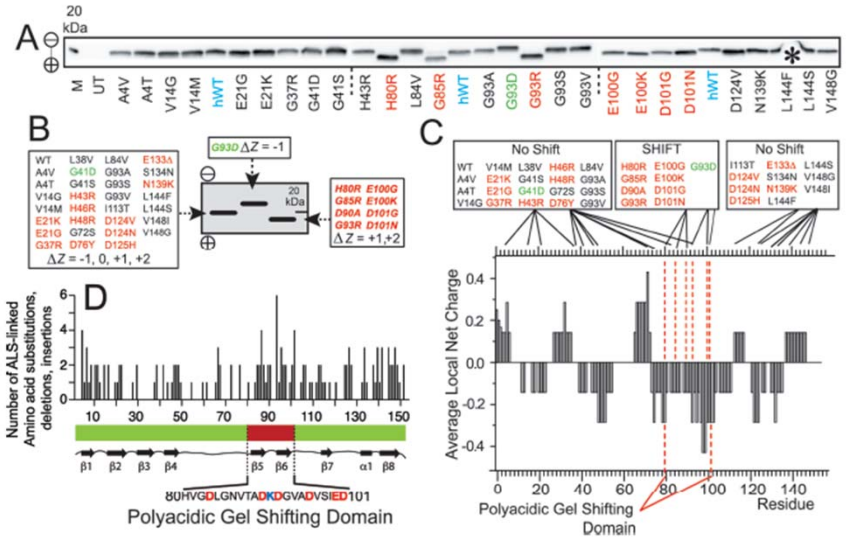
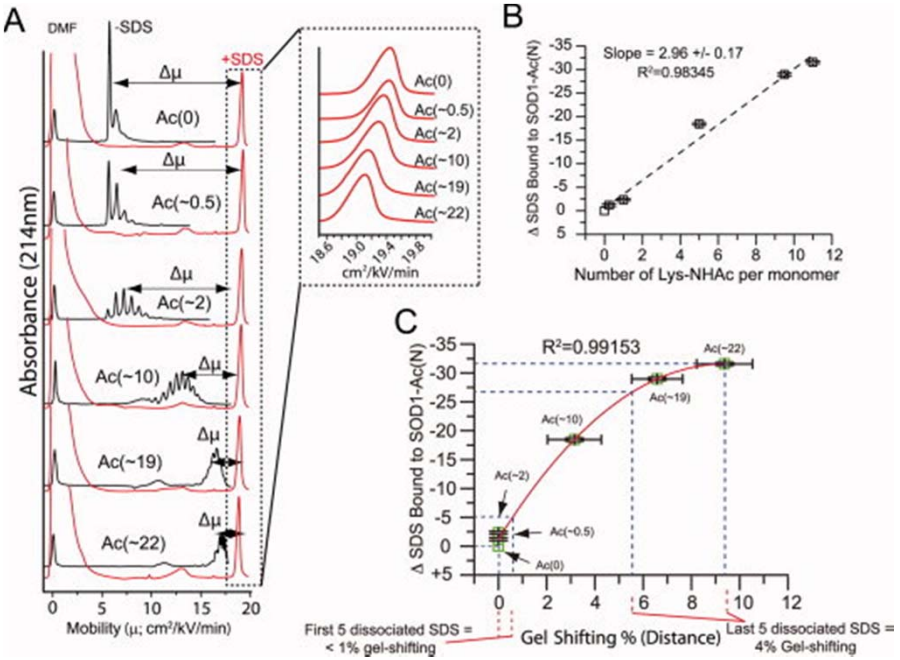
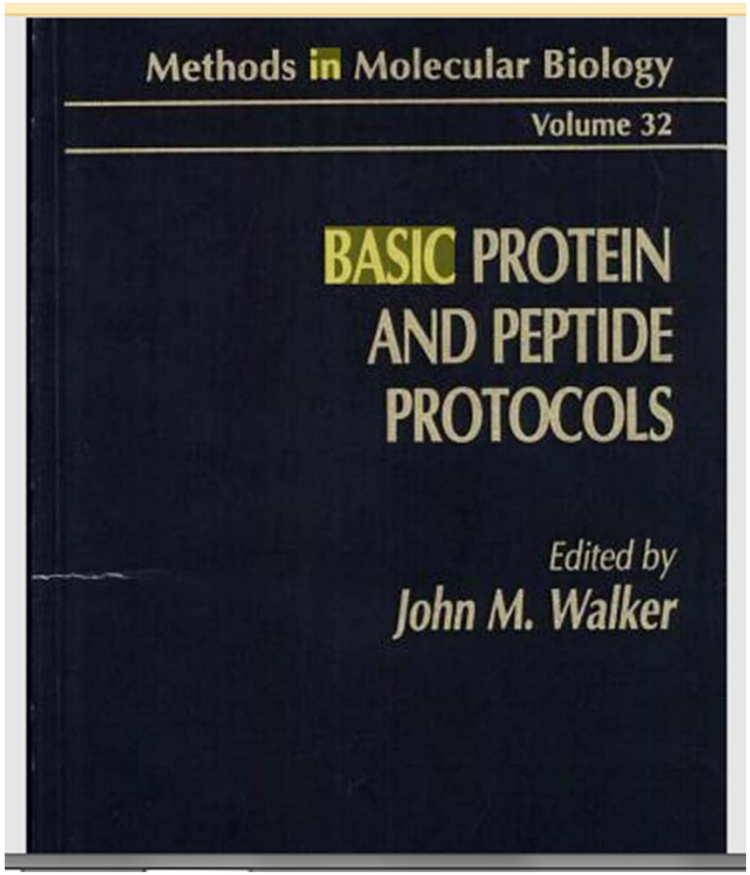


Figure 1. Gel shifting of ALS variant hSOD1 during SDS-PAGE. A: SDS-PAGE and anti-SOD1 Western blotting of ALS mutant hSOD1. Black = no change in migration towards positive electrode; red = increase; green: decrease. Untransformed (UT) cells and cells expressing WT hSOD1 were loaded as controls. Asterisk denotes smearing artifact of Western blotting. Image represents composite of three Western blots (borders indicated by vertical dashed lines). B: Summary of migration of 39 ALS variants of hSOD1 with SDS-PAGE from this study, and published reports. Substitutions that decrease the net negative charge of hSOD1 ($\Delta Z = +$) are highlighted in red; substitutions that increase the net negative charge ($\Delta Z = -$) are green; isoelectric substitutions ($\Delta Z = 0$) are black. C: Substitutions that cause gel shifting (indicated with red dashed lines) are clustered in a polyacidic domain (approximately residues 80–101) which has a high local net negative charge. D: Comparison of location of gel shifting domain with native 2° structure and number of known ALS amino acid substitutions at each residue in hSOD1.



Decreasing the number of Lys-NH_3^+ by acetylation decreases the amount of bound SDS

Basic Proteins: a world apart?



Use of Dynamic light scattering to characterize and optimize buffer conditions

Reprint from

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Biotechnology from bench to business

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OMICS | Drug Discovery | Clinical Research & Diagnostics | Bioprocessing | Biobusiness

Drug Discovery Tutorial

Characterizing Stable Protein Formulations

Detecting and Quantifying Aggregation Using Dynamic Light Scattering

Sigrid Kuebler

Sigrid Kuebler is an application scientist at Wyatt Technology. Web: www.wyatt.com. E-mail: skuebler@wyatt.com.

advantage of SEC is the potential loss

Figure 3. Particle sizes for different buffer conditions for protein preparation A

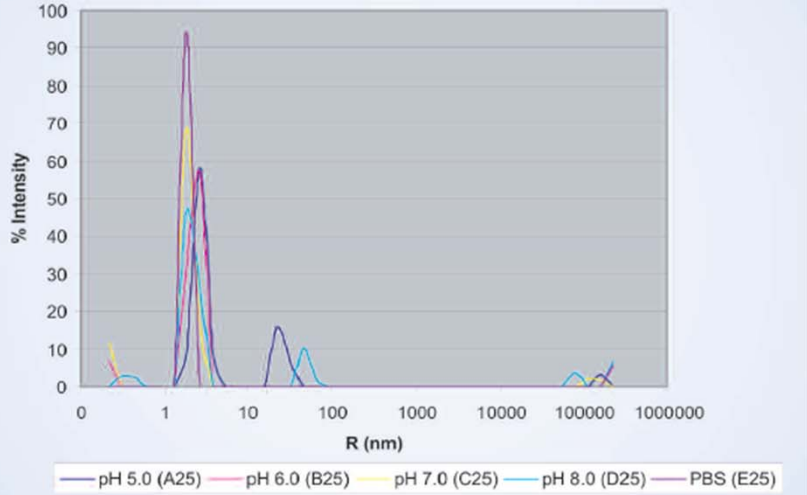
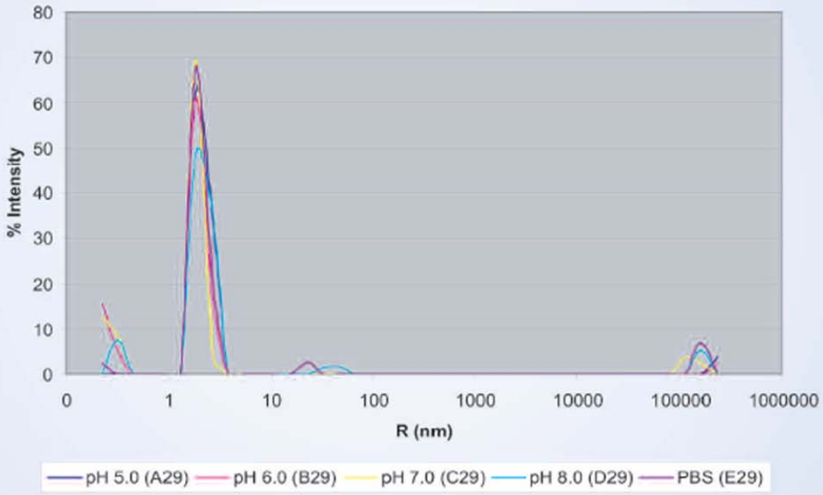


Figure 4. Particle sizes for different buffer conditions for protein preparation B



advantage of being a rapid measurement

The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability

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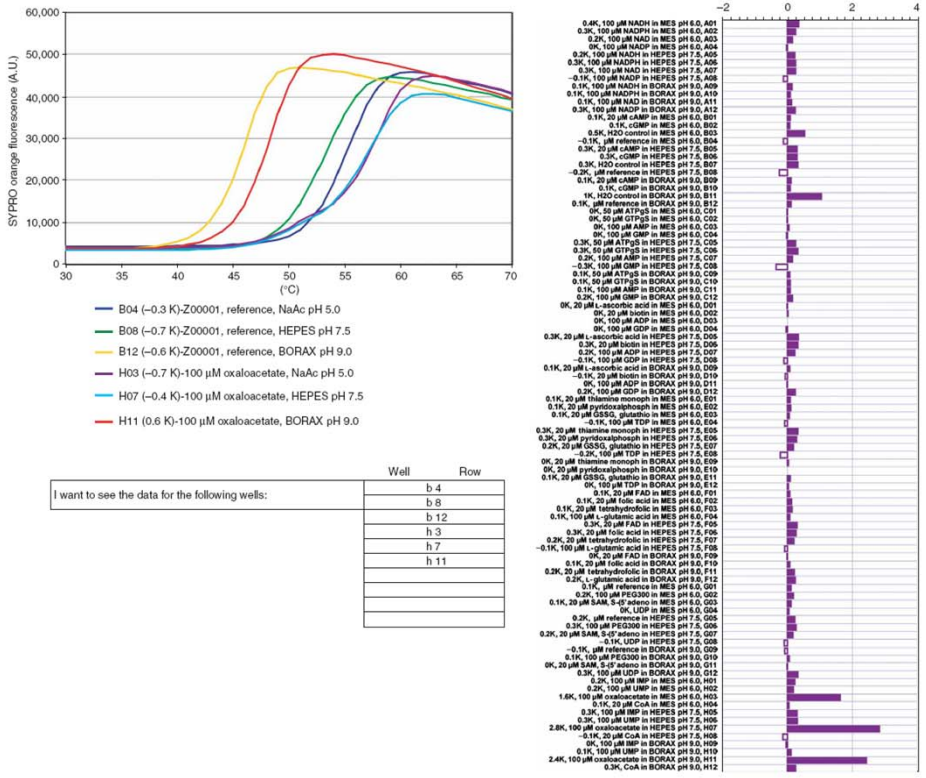

Figure 6 | Visualization of the screen results for citrate synthase against physiologically relevant compounds. Porcine citrate synthase (1 µM) was screened against a selection of 29 compounds (**Table 3**), under three different buffer conditions (entry numbers 4, 8 and 13 in **Table 1**; pH 6.0, 7.5 and 9.0, respectively). The figure depicts a screen shot of two features provided with the DSF analysis tool (ftp://ftp.sgc.ox.ac.uk/pub/biophysics), showing the selection of up to ten curves (left panel) and an overview of tested conditions regarding the ΔT_m values.

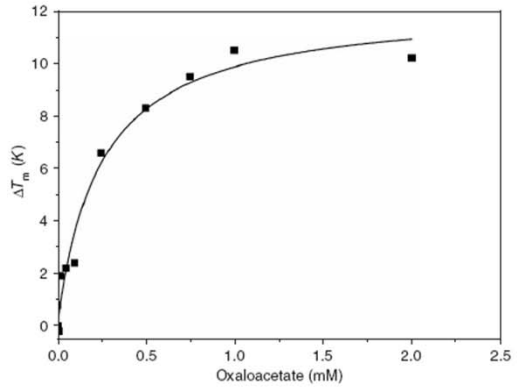

Figure 5 | Concentration-dependent stabilization of citrate synthase by oxaloacetate. The T_m for 1 µM of protein in 10 mM HEPES-NaOH pH 7.5, containing 100 mM NaCl, was measured by DSF at oxaloacetate concentrations between 10 and 2,000 µM. The curve represents an exponential fit to the ΔT_m values plotted against the compound concentration.