Structural Elucidation

13 October 2015 10:04

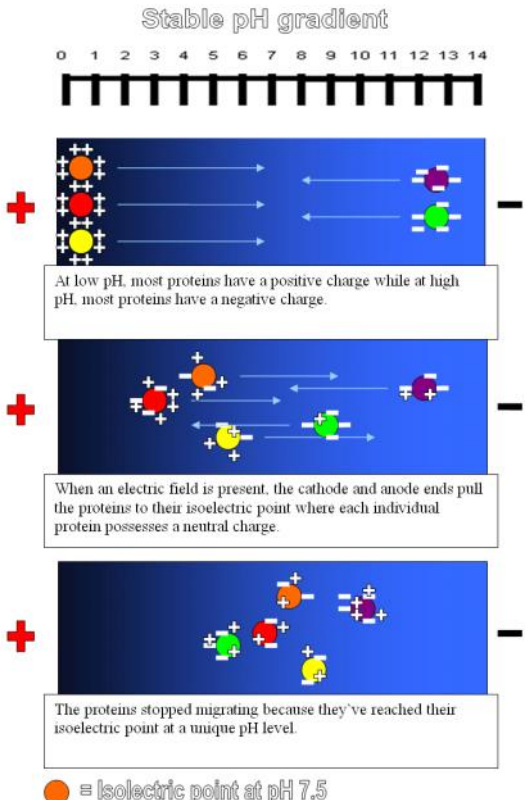
Amino Acid Composition

- Hydrolysis in 6M HCl at 110°C for 24hr
  - Destroys tryptophan
- 2-4M NaOH at 100°C for 4-8 hours
  - Destroys cys, ser, thr, arg
- Amino acids in the hydrolysed mixture are separated by high performance liquid chromatography on an ion exchange column
  - Dowex 50 exchange column
  - Retention time is characteristic to each amino acid
- Amino acids are reacted with dye - ninhydrin
  - Amino acids stain blue (except yellow proline - can only bind 1 ninhydrin)
  - Product has large conjugate system of single/double bonds
  - Can detect 1microgram of an amino acid (amount in a thumbprint)
- Fluorescent staining
  - Fluorescamine
  - Picomolar quantities

Analysis

- Separate from other cellular constituents
  - High salt concentration precipitation
  - Size exclusion chromatography
  - Breakdown tertiary structure (SDS)
  - Break linkages - reducing agents
- Separation from other proteins
  - SDS gel electrophoresis - by size
  - Isoelectric focussing - by PI point
- Quantification
  - Lowry Assay - Folin Ciocalteau reagent. Reacts with phenolic residues
  - Bradford assay - coomassie blue dye

Coomassie Brilliant Blue is the name of two similar triphenylmethane dyes that were developed for use in the textile industry but are now commonly used for staining proteins in analytical biochemistry. Coomassie Brilliant Blue G-250 differs from Coomassie Brilliant Blue R-250 by the addition of two methyl groups. The name "Coomassie" is a registered trademark of Imperial Chemical Industries.

- Isoelectric Focussing
  - Charged protein migrates down gel to point of neutral charge
    - Balance between dissociation of acid and basic groups
  - Every protein has a different number of acid/basic groups
    - PI point is balance
  - 

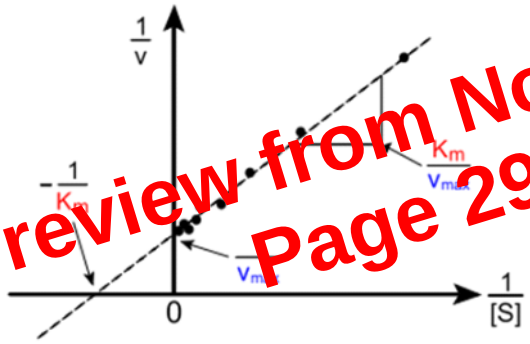
- $V_o = V_{max} \frac{[S]}{K_m + [S]}$

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

- **Using K_m**
  - When [S] = K_m
    - $V_0 = V_{max} \frac{Km}{Km+Km}$
    - $V_0 = \frac{1}{2} Vmax$
    - So K_m is the [S] that gives 1/2 maximal rate
    - Lower the K_m the better the enzyme works at low [S]
- **Importance of K_m**
  - Measure of [S] required for adequate catalysis
  - Enzyme isoforms can have different K_m and V_{max} to suit role
    - Liver glucokinase has high K_m - active at high blood glucose concentration
    - Hexokinase isoform in cells has low k_m - active at normal cell glucose concentration

- **Measuring V_{max} and K_m**
- Can be derived from catalysis rates over a range of [S]
- M-M plot is asymptotic and hard to get a good measure of V_{max} from
  - What is the max value?
- Transformation into a reciprocal M-M plot gives a Lineweaver-Burke plot

$$\frac{1}{V_0} = \frac{K_M}{V_{max}} X \frac{1}{[S]} + \frac{1}{V_{max}}$$



Turnover Number

- $K_{cat} = K_2$
- Number of molecules catalysed per second
- From V_{max}

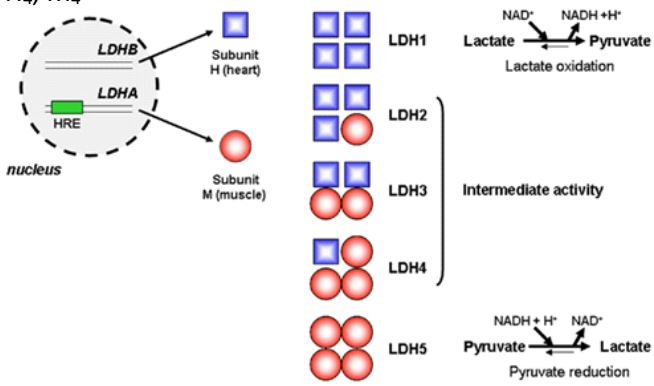
  $V_{max} = K_{cat}[E]_T$

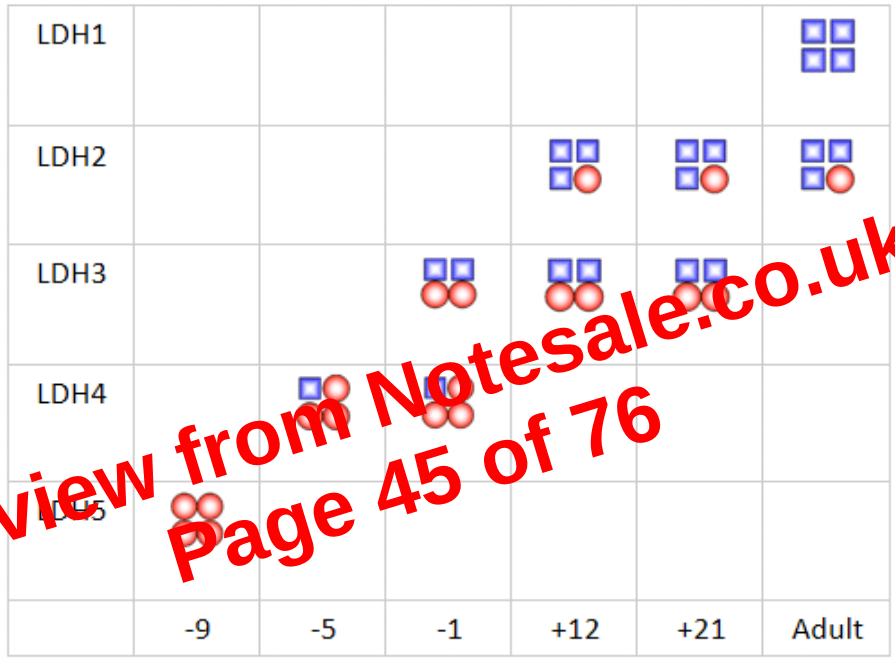
  $K_{cat} = V_{max}/[E]_T$
- How much substrate E can turn to P at max rate
- K_{cat}/K_m is a measure of catalytic efficiency - guide to efficiency under physiological conditions
  - Normally enzymes not saturated
  - $[S]/K_m$ typically 0.01-1.00
  - When $[S]<K_m$ enzymatic rate$<K_{cat}$

    $V_0 = \frac{Kcat}{Km}[E][S]$
  - Increase efficiency by increasing V_{max} or decreasing K_m

How fast can an enzyme go?

- V_{max} cannot be faster than diffusion-controlled encounters of E and S
- Diffusion limits catalytic efficiency (K_{cat}/K_m) to $10s^{-1}M^{-1}$
- For catalytically perfect enzymes every encounter of E and S goes to P

- Lactate dehydrogenase
  - Anaerobic glucose metabolism/glucose synthesis
  - Humans have 2 isozyme subunits
    - H_4, M_4
    - 

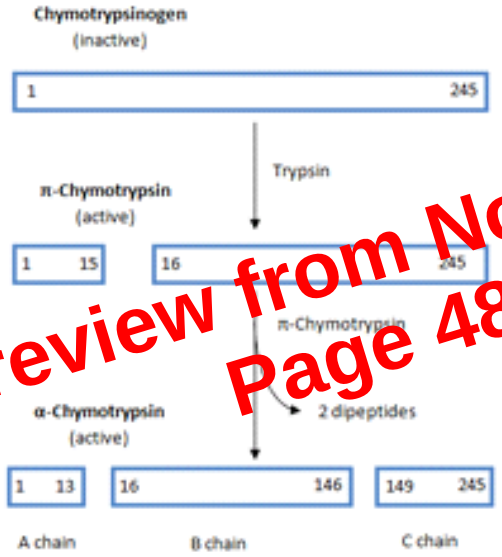
    - High similarity
    - Tetrametric enzyme - combinations
    - M works optimally in anaerobic conditions, H works optimally in aerobic conditions
  - Make up of LDH differs between developmental stage and organs
    - 

  - LDH content varies by tissue
  - Appearance in blood indicative of damage

3. **Reversible Covalent Modification**
   - Covalent attachement
     - Another moelcule modifies enzume activity
   - Donor molecule
     - Functional moiety
     - Enzyme properties
   - Most common types
     - Phosphorylation
     - Acetylation
   - Regulation of acetyltransferase and deacetylase
     - Regulated by phosphorylation
   - Most are reversible
   - Phosphorylation
     - Most common regulatory mechanism
       - Intracellular proteins
       - 30% eukryotic protons phosphorylated
       - Reversible

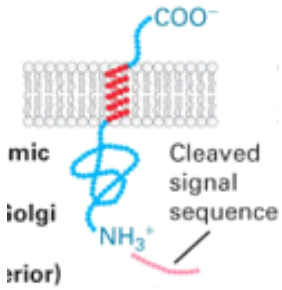
        - Coordination
    - Determination of specificity
        - Sequence surrounding Ser or Thr
        - Consensus
            - Arg-Arg-X-(Ser/Thr)-Z
            - Variations
            - Distant residues
        - Activation of protein kinase A
            - PKA - covalent/allosteric
            - Fright, fight, flight reaction
                - Adrenaline
                - Adrenaline triggers cAMP formation
                    - Intracellular messanger
                - cAMP activates PKA
                    - Altering of 4° structure
                    - Phosporylation
            - PKA is a holoenzyme - subunits R (regulatory) and C (catalytic)
                - Without cAMP R_2C_2 structure
                - cAMP causes dissociation to R_2 and 2xC
                - R chain pseudosubstrate - blocking C active site

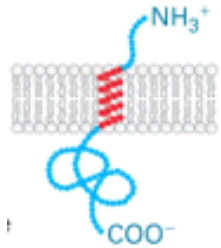
4. **Zymogen**
    - Enzyme activity acquired
        - 3D folding
    - Folded forms of transcribed enzyme
        - Inactive
        - Zymogens
    - Activation
        - Peptide cleaving
    - Permanent
        - Proteolytic deactivation.l
    - Digestive enzymes
        - Pepsinogen --> pepsin
        - Trypsinogen --> trypin
        - Proelastase --> elastase
    - Zymogen systems
        - Blood clotting
            - Cascade of zymogen conversions
            - Rapid response
        - Hormones
            - Transcribed as zymogens
            - Proinsulin - activated by cleavage
        - Collagen
            - Major bodily constituent
            - Transcribed as procollagen
        - Developmental stages
            - Metamorphosis

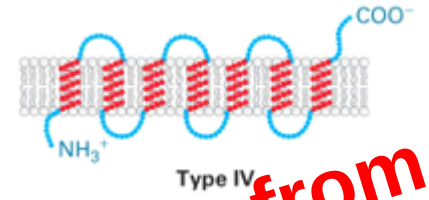
        - Parturition (uterus post-birth)
        - Rapid breakdown of collage
        - Procollagenase
    - Apoptosis
        - Programmed cell death
        - Conversion of procaspases
- Chymotrypsinogen Activation
    - Synthesised by pancreas
        - Acinar cells
        - Membrane bound granules
    - Single polypeptide chain
        - No enzymatic activity
    - Converted to pi-chymotrypsin
        - Arg15-Ile16 cleaved (by trypsin)
            - N-terminal Ile16 turns inwards, forms ionic bond with Asp194
            - Electrostatic changes causes met192 to move to the surface
            - S1 pocket formed (specificity)
            - Shifts form oxyanion hole
    - Pi-chymotrypsin acts on itself
        - Dipeptides (146 and 149, 13 and 16)
    - Results
        - Fully activated alpha-chymotrypsin
        - Three polypeptide chains linked by disulphide bridges
    - 

- Common activators
    - Uidenum - concurrent activation
    - Digestion of proteins and blood clotting
        - Switching numerous enzymes/proteins simultaneously
    - Common activator
        - Coordinate control
    - Food enters duodenum
        - Enteropeptidase
        - Hydrolysis lys-Ile bond in trypsinogen
    - Pancreatic trypsin inhibitor
        - Zymogen activation irreversible
        - Lies in active site
            - S1 pocket
            - Maximum inhibition
        - Other inhibitors
            - Inhibit common activator
            - Rapid enzyme turnover

- Hsp90 - steroid hormone receptors
  - Regulatory functions
  - Increased during stress
  - Cytosolic and ER forms 82-94kDa
  - Reversible binding with target acts as on/off switch
  - Regulates wide range of functions

- 

- Type II
  - Asialoglycoprotein receptor, transferin receptor
  - COO^- inside, NH_3^+ outside
  - Enter through translocon
  - Signal peptide within sequence - signal anchor
  - Anchored within membrane and extruded sideways
  - 

- Type IV
  - Multiple loops/helices through membrane
  - COO^- or NH_4^+ on either side
  - 

    Type IV
- Determined by location and order of stop transfer, signal, anchor segments
- Hydrophobic: Leucine L, Isoleucine, Valine V
  - Hydrophobicity plot: +ve = more hydrophobic

Mitochondrial Uptake

- Proteins synthesised in cytosol
  - Uptaken if mitochondria then added (unlike ER)
- Translocons at each membrane
  - Translocons conjugate to 'skip' intermembrane space
  - Chaperone proteins within membrane

Experimenting with Proteins

24 November 2015 10:06

Solving Structures

24 November 2015 10:06

Protein Structure Determination

- 2 main methods
  - NMR spectroscopy
  - X-ray crystallography
- x-ray crystallography requires protein to be crystalline
  - Very difficult
  - Most proteins don't crystallise
  - 39,000 structures mapped
- NMR can be performed on proteins in solution
  - Data analysis very hard
  - 6,000 structures mapped

How to determine structure

- Primary methods
  - x-ray crystallography
  - NMR spectroscopy
  - Cryo-electron microscopy
- Secondary methods
  - Circular dichroism
  - Fluorescence
  - Neutron scattering

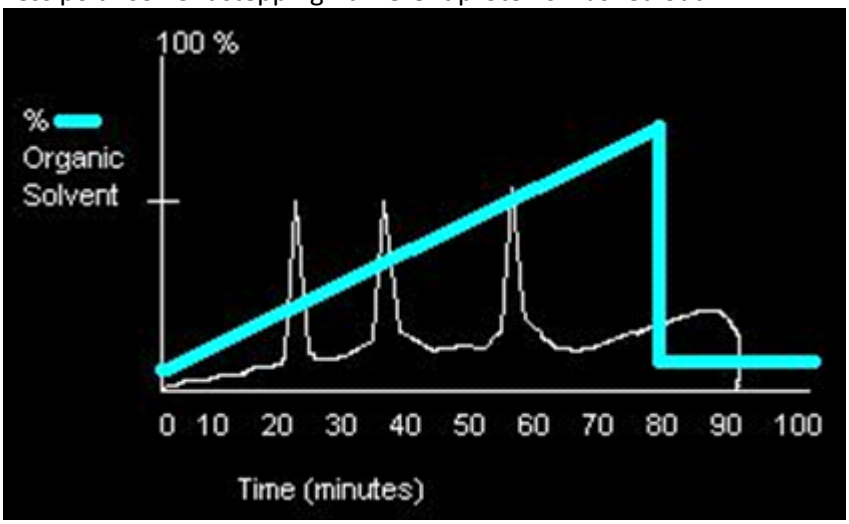
Cryo-Electron Microscopy

- Sample flash frozen - liquid ethane
- Solvent not given time to form ice crystals
  - Obtain snapshot of sample in solution
- 2 approaches
  - 2D crystal
    - 2D layer forms
      - Tilted and snapshots taken
      - 3D image built up from different angles
  - Image reconstruction
    - Proteins don't always form flat layer - random aggregations
    - Many photos taken at different angles
    - Common features used to identify the same regions
    - Can be wrong, giving incorrect proteins

How Accurate are the structures?

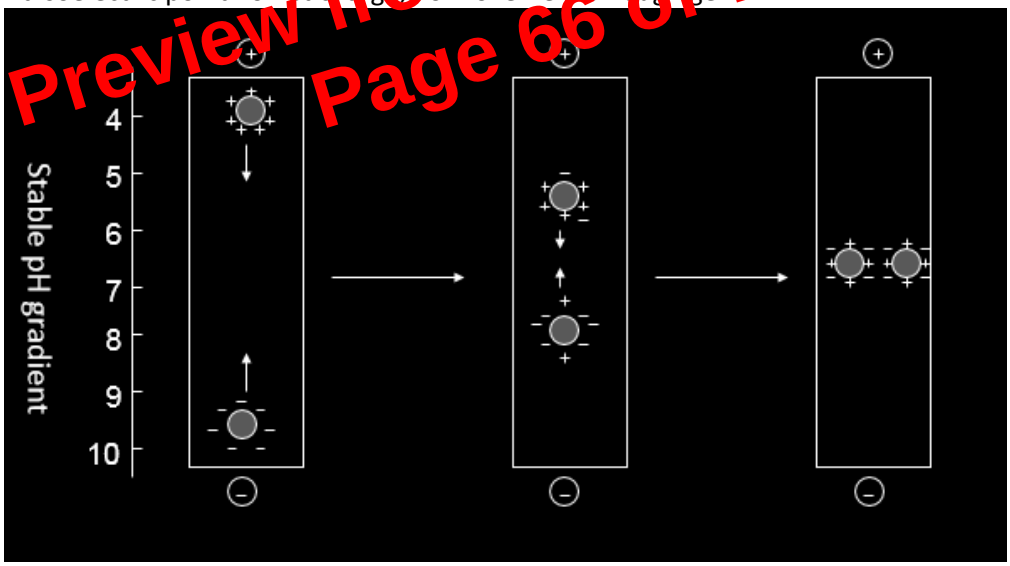
- Resolution of structure
  - Measure of how many data collected
- More data - greater ratio of observations to number of atomic coordinates to be determined - in principle the greater accuracy
- Resolution in Angstroms
  - Lower number greater accuracy and level of atomic detail
- Hydrogen atoms visible at 1.5Å
- 2Å enough for orientation of peptide chains

Structural feature/ Resolution.	5 Å	3 Å	2.5 Å	2 Å	1.5 Å
Chain tracing	-	Fair	Good	Good	Very good
2nd Structure	Helices fair	Fair	Good	Good	Very good
Side chain	-	-	Fair	Good	Very good

        - Mobile phase
        - Polar
        - Contains proteins
    - Non-polar protein favours stationary phase
    - Polar washes out in solvent faster
    - Less polar solvent stepping - different proteins washed out
    - 

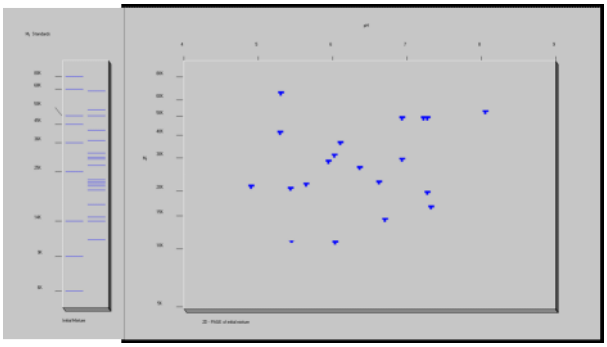
- NP
    - Non-polar mobile phase
    - Polar stationary phase

Isoelectric Point

- pH gradient
- Current run through
- Proteins move according to charge
    - Positively charged move to negative end
- pH alters charge of proteins
- At isoelectric point no net charge, no movement through gel
- 

Ion Exchange Chromatography

- Anion exchange
- Immobilised cation surface
- Mix of amino acids carried through in solution
- Negative residues immobilised on surface
- Positive residues carried through with solution
- Can change pH to alter charge and release
- Can use positive resin

- 

2D Staining and Analysis

- Coomassie >75ng
- Silver staining >5ng
- Fluorescent staining >5ng

Zymography

- Impregnate gel with substrate
- Run gel
- Incubate at appropriate temp (e.g. 37°C)
- Bands of clearing
  - Staining coomassie blue
  - Active proteins digest
- 

Quantifying

- Software identifies and compares spots

Western Blotting

- Run gel
- Transfer to membrane
- Attach antibodies
  - Target protein
- 2° antibdy with enzyme
  - Colour change

        - GST agarose
        - s-hexyl glutathione agarose
        - ^ both glutathiones bound to beads
        - GST bound to residues
            - Elute with glutathione solution
        - Purified and separated GSTs
    - As a mixture of GSTs, could not use enzyme activity to confirm presence of Mu class
        - Western blotting - Mu class antibody
    - Used sequence information to confirm
        - Genome unknown at the time
            - Small transcriptome only
        - Tandem mass spectrometry - fragments of segments, weight of a.a. residues
        - Did TMS for each blot spot
        - Mu class and sigma class. Sigma class not identified before.

Sigma GSTs

- Produced more
    - Recombinant DNA into *E. coli* - expressed sigma GST
    - Purified
- In situ expression
    - Got antibodies by infecting rabbits
    - 1D gel, probed with anti-GST antibodies
        - Heavily expressed in eggs and juvenile, present in adult and adult excretion

Immuno-localisation

- Liver and bile ducts
- Testes free of protein
- Surface free (but near)
- Heavily in ovaries/eggs

Function?

- Activity rFhGST-S1
    - Model system substrates
    - Table 1. Substrate specificity of rFhGST-S1.

GST Class	SUBSTRATE	[Co-substrate] (mM)	[GSH] (mM)	100 mM $KHPO_4$ (pH)	Temp. (°C)	λ Max (nm)	ε ($mM^{-1}cm^{-1}$)	rFhGST-S1 Specific Activity (nmol $min^{-1}mg^{-1}$)	Sm28GST** Specific Activity (nmol $min^{-1}mg^{-1}$)	Assay Ref.
MODEL SUBSTRATES	1-Chloro-2,4-dinitrobenzene (CDNB)	1	1	6.5	25	340	9.6	4736±292	7269±218	[36]
	1,2-Dichloro-4-nitrobenzene (DCNB)	1	5	7.5	25	345	9.6	ND<5	ND<5	[36]
	Ethacrynic Acid	0.08	1	6.5	25	270	5	898±204	1580±97	[70]
REACTIVE ALDEHYDES	4-hydroxynonenal	0.1	0.5	6.5	30	224	13.75	645±129	287±17	[68]
	Trans-2-nonenal	0.023	1	6.5	25	225	−19.2	333±43	447±6	[36]
	Trans, trans-2,4-decadienal	0.023	1	6.5	25	280	−29.7	51±0.3	221	[36]
LIPID PEROXIDES	Cumene hydroperoxide	1.2	1	7	25	340	6.22	7081±1009	162±7	[71]
		0.25	1	7	25	340	6.22	2209±122	-	[71]
	t-butyl hydroperoxide	0.25	1	7	25	340	6.22	193±1.8	ND<10	[69]
		2.5	1	7	25	340	6.22	1827±198	-	[69]
	Linoleic Acid	0.25	1	7	25	340	6.22	430±69	-	[51]
		0.05	1	7	30	340	6.22	-	ND<10	[51]

Recombinant FhGST-S1 shows activity towards a broad range of model and natural GST substrates with a similar enzymatic profile to the Schistosomiasis vaccine trialist (Sm28GST - P09792). rFhGST-S1 also displays high glutathione-dependent lipid peroxidase activity compared to both Sm28GST and Sj26GST (Q26513) [47]. Reasonably high GSH-dependent lipid peroxidase activity has also been seen in a 'weak affinity' fraction following chromatofocusing of GSH transferase activity that failed to bind GSH-sepharose [10]. ND – Not determined.
**Data taken from Walker *et al.* [47].
doi:10.1371/journal.pntd.0001666.t001

    - Lipid peroxide metabolism
        - Detoxification of worm
    - Inhibition (conc. For 50% inhibition)
        - TCBZ 57µM - higher than Mu class (lower affinity), probably not directly involved in drug detox
        - Bile acid 64-302µM - lower affinity than Mu again
- Structure
    - x-ray crystallography
        - Ligands