Antioxidant Enzymes and Function

I. Superoxide Dismutase (SOD) - 1968-1969, McCord and Fridovich (JBC)

A. Function

$$O_2^{\bullet -} + O_2^{\bullet -} + 2\ H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (k_{catalytic}\ 2\text{-}4 \times 10^9\ M^{-1}s^{-1})$$

Only enzyme known to act on a radical.

Revolutionary idea: the presence of SOD implies $O_2^{\bullet -}$ produced in cell during normal metabolism.

*Note - SOD is a **primary** antioxidant enzyme - acts on a ROS

What is unique about SOD?

B. Forms

Differences:

1. a.a. sequence
2. active metal site
3. cellular location

Table 1 Procaryotic Cells - SOD

	MW/Da	Subunits
FeSOD	40,000	2 dimer
MnSOD	40,000	2 dimer
	80,000	4 tetramer

Table 2 Eucaryotic Cells - SOD

	MW/Da	Subunits
MnSOD	88,000	4
CuZnSOD	32,000	2
EC (CuZn) SOD	135,000	4
EC MnSOD	150,000	2,4

EC = extracellular

C. Intracellular Location of SOD's

a. Procaryotes:

MnSOD - matrix (inner)

FeSOD - outer membrane

b. Eucaryotes:

CuZnSOD - cytoplasm, nucleus, lysosomes

MnSOD - mitochondrial matrix

EC(CuZn) SOD - plasma membrane, extracellular

ECMnSOD - plasma membrane

D. Structure and Properties

a. CuZnSOD

Largely, acidic proteins pI 4-6. 150 - 155 a.a. residues per SOD subunit

- MW = 32,000 Da, dimer

- high glycine, low tyrosine & tryptophan

Stability

* One of the most stable proteins

Not dissociated by SDS alone (breaks apart H bonds)

Disassociated by: SDS + β-mercaptoethanol or EDTA + heat 40-55°C

$T_{1/2}$ = temperature to break apart 50% in 10 min = 67°C

Activity is constant from pH 4.5 to 9.5

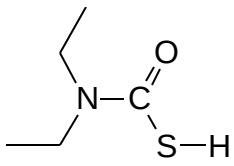
Stable to repeated freeze thaw cycles and to prolonged refrigeration.

Inactivated by:

6 M guanidine hydrochloride

1-5 mM NaCN (cyanide binds copper)

1-5 mM DDC, diethyldithiocarbamate (binds copper)



NaCN and DDC are used to inhibit CuZnSOD, but do not affect MnSOD

b. 3-D Crystalline Structure of CuZnSOD from Bovine Erythrocytes

Cu and Zn are 6 Å apart
2 Cu on separate strands are 34 Å apart
Zn binds 3 His and 1 Asp (His 61, His 69, His 78, Asp 81)
Cu binds 4 His - active site (His 44, His 46, His 61, His 118)

Imidazole of His 61 lies between Cu and Zn

Representative of polypeptide structure of bovine CuZn SOD
Oberley, L.W. Superoxide Dismutase, Vol. I, p. 28, 1982.

c. Chemical Modifications of CuZnSOD

H_2O_2 - limits activity

At low concentrations change cupric (Cu^{2+}) to cuprous (Cu^{1+}) and cause reversible inactivation.

High concentrations or long exposures cause irreversible inactivation.

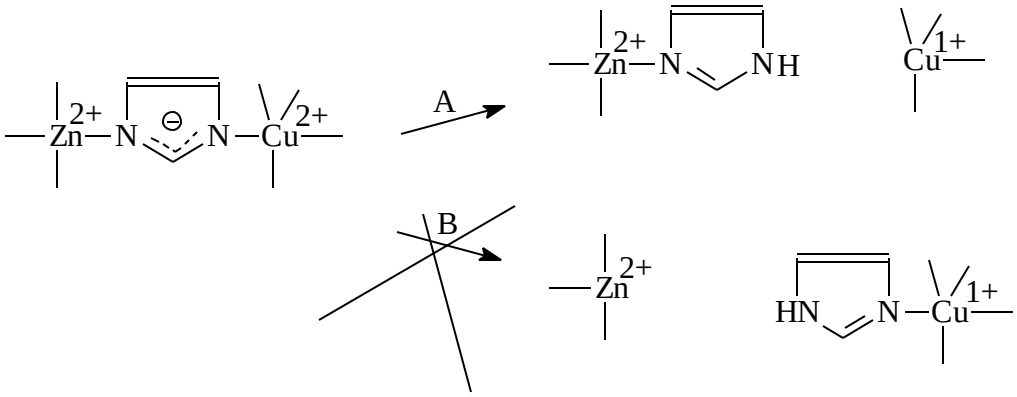
Butanedione and phenylglyoxal inactivate CuZnSOD by modifying arginine residues. Arg 141 in acitve site

d. Catalytic Mechanism (E = enzyme)

$E\text{-}Cu^{2+} + O_2^{\bullet-} \rightarrow E\text{-}Cu^{+1} + O_2$ electron transfer

$E\text{-}Cu^{1+} + O_2^{\bullet-} + 2H^+ \rightarrow E\text{-}Cu^{2+} + H_2O_2$ proton and e^- transfer

Bridging Imidazolate Hypothesis - A is correct



e. ECSOD - Markland, 1982

slightly hydrophobic glycoprotein

MW = 135,000 Da

4 equal, noncovalently bound subunits

4 Cu and 4 Zn

Inhibited by cyanide, azide, H_2O_2, DDC, SDS

Three fractions, according to binding of heparin

Sepharose: A, no affinity; B, weak affinity; C, high affinity.

240 amino acids per subunit; 18 a.a. are signal peptide

MW = 24,174 Da

N-glycosylation site (Asn 89)

First 95 a.a. show no sequence homologies with CuZnSOD

From His 96 to Gly 193, ECSOD has strong homology to CuZnSOD. 49 of 76 positions are identical.

ECSOD shares amino acids in 22 of 23 positions in which CuZn is invariant.

All ligands to Cu (His 96, His 98, His 113, His 163) and Zn (His 113, His 121, His 124, Asp 127) are found in CuZn and ECSOD acitve site.

Cys 107 and Cys 189 forming intrasubunit disulfide bridge found in both proteins.

Arg 186 found in both.

Hjalmarsson, et al. *Proc. Natl. Acad. Sci. USA*, p. 6343 1987.

Carboxy-terminal end of ECSOD is very hydrophilic and contains (+) charged a.a.

Marklund proposed this part binds heparin.

SDS gels - MW= 32,000 Da and 29,500 Da

Binds to ConA, lentil, wheat germ lectins. Shows are glycoproteins.

Biological

Major SOD in extracellular fluids such as plasma, lymph, and synovial fluid. Also found in tissues. Binds to endothelial cells in vasculature. Involved in inflammation.

Heparin suppresses inflammation by releasing ECSOD!

f. Fe/MnSOD

Fe/MnSOD generally dimers

Tetramers found in:

Most MnSOD from eukaryotes

MnSOD from some bacteria

FeSOD from one bacteria

Trimer MnSOD found in one bacteria.

E. coli FeSOD (MW 21,111 Da) 192 a.a.;
Human MnSOD (MW - 22,200 Da/monomer) , monomer 196-198 a.a. – a tetramer

Most Mn/FeSOD are acidic proteins with pI 4-5

Stability

In general, not as stable as CuZnSODs. Subject to freeze thaw inactivation

As pH increases, SOD activity goes down; greater than pH 7.8 activitiy decreases.

Mn/FeSOD only slowly inactivated by CN; FeSOD inactivated by H_2O_2.

Metal reconstitution studies:

Many divalent metals bind to the active sites. Only Mn gives activity to MnSOD and only Fe to FeSOD and not vice versa. Resting metal states present as Mn^{3+} and Fe^{3+}.

g. Synthesis of MnSOD in eukaryotic cells

Human liver (Wispe, *BBA* **994**:30036, 1989.)

1. MnSOD is encoded by nuclear chromatin;

2. mRNA migrates to cytosol

3. Protein is made in ribosome

Made as a precursor form with a MW = 26,000 Da

The precursor is imported post-translationally into mitochondrial matrix.

Precursor is clipped by protease in inner mitochondrial membrane to 24,000 Da protein.

Proteolytic processing is accompanied by energy dependent import through the membrane.

* Anything blocking ATP formation blocks MnSOD uptake

* Blocked by CCCP and NaCN

II. Catalase (CAT)

History

Thernard, discoverer of H_2O_2, first noted in 1818 that animal tissues could decompose H_2O_2.

Loew in 1901 introduced the name catalase for the natural compound that decomposes H_2O_2.

Wolft and de Stoecklin achieved first hemoglobin-free purification in 1910.

A. **Functions:**

a. Enzymatic Functions

1. Catalytic

$$2\ H_2O_2 \xrightarrow{CAT} 2\ H_2O + O_2$$ * note oxygenates

2. Peroxidative first substrate is H_2O_2

$$ROOH + HQOH \longrightarrow QO + ROH + H_2O$$

A. $H_2O_2 + AH_2 \rightarrow A + 2\ H_2O$

B. $ROOH + AH_2 \rightarrow H_2O + ROH + A$

b. Biological Functions

1. Removes H_2O_2, adds O_2

2. Protects against lipid peroxidation

3. May participate in alcohol metabolism

4. In bacteria, low CAT mutants are hypersensitive to H_2O_2.

   In Drosophilia, null mutants age faster

B. **Location & Forms**

There are many forms of CAT. Most contain Fe - heme, but some contain Mn.

Most anaerobic bacteria do not contain CAT, most aerobic bacteria contain CAT.

a. E. Coli 2 CAT

1. HPI - periplasmic membrane

   - tetramer MW 337,000

   - 2 molecules of protoheme IX per tetramer

   - bifunctional: catalytic or peroxidative

   - inducible by H_2O_2 or Ascorbate

   - Increase during log growth

2. HP II - cytoplasmic

   - tetramer

   - 2 molecules of protoheme IX per tetramer

   - monofunctional - peroxidatic activity only

   - not inducible by H_2O_2 or Ascorbate

   - Increases during stationary phase of growth

b. Maize - 3 CAT found in different cells and expressed differentially during development

   All are tetramers of MW 240,000

   Each one or two amino acids different

c. Human At least 2 forms. Found in cytoplasm and peroxisomes.

One report found CAT in cytoplasmic granules of eosinophils.

J. Histochem. Cytochem. **30**:697, 1982.

Tissues -Most in liver (hepatocyte, peroxisomes) and erythrocyte (cytoplasm)

Some found in brain, heart, skeletal muscle, and kidney

Heart CAT found in mitochondria. B. Freeman, 1991.

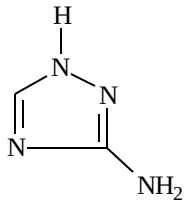
C. **Structure and Properties**

Typical catalase has 4 identical subunits, each with a heme in active site.

Different catalases may have additives or deletions of C-terminal amino acids.

Molecular Weights/Da	
Micrococcus lysodeikticus	232,000
Yeast	240,000
Horse liver	225,000
Human blood	220,000

Catalase inhibitor - 3-amino-1, 2, 4- triazole covalently binds His 74, requires H_2O_2 to inactivate.



D. **Catalytic *vs.* Peroxidative**

Overall: $ROOH + HQOH \rightarrow QO + ROH + H_2O$

R = H, acyl, aryl

1st step:

$E\text{-}OH_2 + ROOH \rightarrow E\text{-}O + ROH + H_2O$

resting Cmpd I

2nd step:

$E\text{-}O + HQOH \rightarrow E\text{-}H_2O + QO$

Q = Oxygen catalytic $k = 10^7$ L mol $^{-1}s^{-1}$

Q = (C = 0) or 1-3 carbon chain peroxidative $k = 10^2 - 10^3$ L $mol^{-1}s^{-1}$

Kinetics CAT Fe(III) + H_2O_2 $\xrightarrow{k_1}$ Cmpd I

Cmpd I + H_2O_2 $\xrightarrow{k_2}$ CAT(FeIII) + $2H_2O + O_2$

$k_1 = 1.7 \times 10^7\ M^{-1}\ s^{-1}$ (= L $mol^{-1}s^{-1}$) fast!

$k_2 = 2.6 \times 10^7\ M^{-1}\ s^{-1}$

Normal kinetics are difficult to do because:

Difficult to saturate CAT with H_2O_2 due to large k's.

H_2O_2 inactivates CAT at concentrations above 0.1 M, when compound I is converted to inactive compounds II or III.

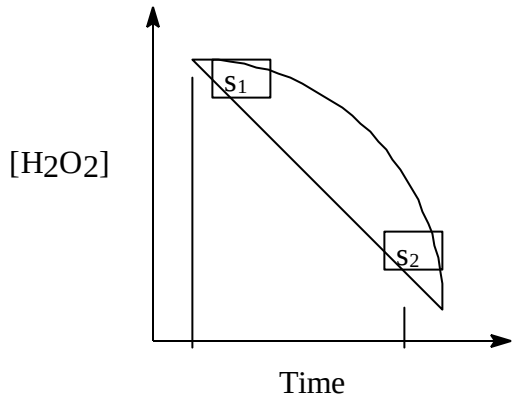
E. Measure catalase by measuring peroxide removal

moles H_2O_2 used ($M \bullet s^{-1}$) = $2\ k_2\ [H_2O_2]$ [Cmpd I]

= $2\ k_1\ [H_2O_2]$ [free catalase]

Fix $[H_2O_2]$ .01 M, then [free CAT] = $\dfrac{\text{moles } H_2O_2 \text{ used } (M \bullet s^{-1})}{2\ k_1\ [H_2O_2]}$

or [CAT] $\propto$ $[H_2O_2]$ used up



exponential, Abs 240 nm, not sensitive

$k = 1/\Delta t \ \ln \ s_1/s_2$ Methods of Enzymology,vol. 105, 121-126.

Role of NADPH

In higher organism - CAT binds 4 NADPH., Tightly bound for example human and bovine catalase

1. protects CAT from H_2O_2 inactivation ?
2. source of NADPH for GPx during stress ?

F. Unusual CAT - (exceptions) *JBC* **238**: 6015-6019, 1985.

Non heme CAT in *Lactobacillus planterum* (has no SOD)

In resting state contains Mn(III); MW= 172,000 ± 4000

6 subunits of MW 28,300 ± 600 daltons

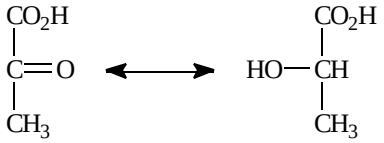
1.12 ± 0.37 atoms of Mn per subunit

Synthetic CAT - Fe cmplx

Liposomal / PEG CAT

Pyruvate - from glycolysis

- reacts stochiometrically like catalase, but not catalytically



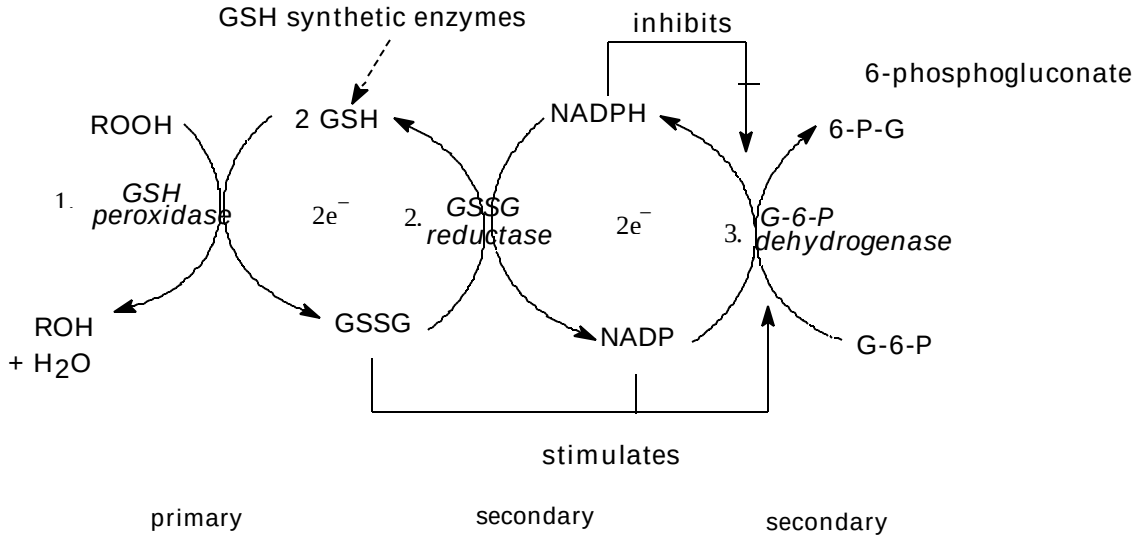
pyruvate L(+)-Lactic acid

acts as CAT binds Mn

acts as SOD

III. Glutathione and Glutathione Peroxidase - rids of H_2O_2 or ROOH (hydroperoxide)

A. General Scheme

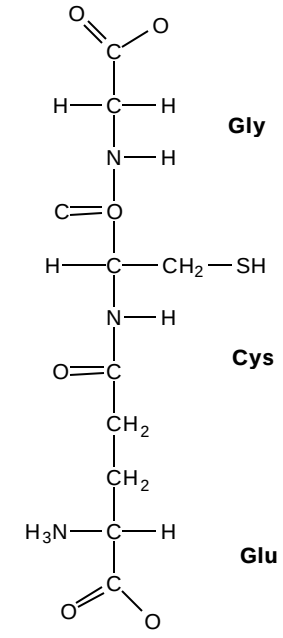


GSH = reduced glutathione; γ - Glu-Cys-Gly, γ- glutamylcysteinylglycine, GSSG = oxidized
Rate limiting enzyme of pentose phosphate cycle is G-6-P dehydrogenase

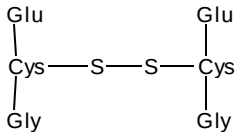
NADP & GSSG both overcome NADPH inhibition of G-6-P dehydrogenase

BSO - buthionine sulfoximine inhibits GSH synthesis

BCNU - inhibits enzyme GSSG reductase (antitumor agent, esp. brain tumors)



glutathione



glutathione

B. Glutathione Peroxidase (GPx)

Discovered by Mills in 1957

A. Function:

a. Enzymatic

$ROOH + 2GSH \xrightarrow{GPx} ROH + H_2O + GSSG$

Unspecific for hydroperoxides. Can be about anything from H_2O_2 to peroxidized membranes and DNA.

Specific for GSH. Similar compounds have much less reactivity.

It yields a single oxidation product, in contrast to heme peroxidases.

b. Biological

Removal of H_2O_2:

- Genetic or alimentary deficiency in GPx suffer hemolytic episodes if exposed to drugs generating $O_2^{\bullet -}$, H_2O_2, or lipid peroxides.

Removal of other hydroperoxides:

- protection against lipid peroxidation

- protection against DNA hydroperoxides

Arachidonic acid cascade

- catalyzes formation of prostaglandins

2. Location and Forms

GPx is not found in bacteria or higher plants, but found in all eukaryotes.

Amounts: high (liver); moderate (heart, lung, brain); low (muscle).

Five known forms:

a. Cytosolic GPx (GPX-1)

Bovine erythrocytes are usually studied. Soluble tetrameric protein of MW = 85,000 Da

Rat liver MW = 75,000 Da

Human erythrocyte = 95,000 Da

Human placenta = 85,500 Da

Equal subunits of MW = 21,000 Da

Each subunit contains a Se. No other metal.

Active site contains a selenocysteine.

b. Mitochondrial GPx- never been isolated, but mitochondria have no CAT, so something must be important for the removal of peroxide. It may be a related enzyme such as thioredoxin/peroxiredoxin.

c. Human Plasma GPx

Tetramer 21.5 to 22.5 kDa per subunit. One Se per subunit.

1529 bp, 226 a.a. Synthesized and secreted by kidney.

Distinct from cytosolic (49% homology) and phospholipid Gpx.

d. "Expression, characterization, and tissue distribution of a new cellular selenium-dependent glutathione peroxidase, $GSHP_X$-GI."*J. Biol. Chem.* 268:2571-2576, 1993.

Tetrameric protein localized in cytosol.
Monomer MW = 22,000, 190 amino acids
Similar substrate specificities as cytosolic GPx ($GSHP_X$-1).

Both reduce H_2O_2, tert-butylhydroperoxide, amino hydroperoxide, and linoleic acid hydroperoxide, but not phosphatidylcholine hydroperoxide.

e. Phospholipid hydroperoxide glutathione peroxidase (PH-GPx; GPx - IV)

First isolated from pig heart in 1982. Active toward hydroperoxides of phospholipids. The other GPx require phospholipase to clip hydroperoxides. Rat liver PH-GPx needs detergent for activity, pig heart does not.

Rat liver - monomer, MW = 22,000 Da

Pig heart - monomer, MW = 20,000 Da

Contains Se. Active site is conserved, but the rest of the protein is quite different. Homology is 25% for plasma EC-GPx and 35% for GPx

(with PH-GPx) in terms of amino acids.

3. Catalytic Mechanism

SeH selenol

SeOH selenic acid

$$E - CysSe^- + H^+ + ROOH \rightarrow E - CysSeOH + ROH$$

selenol → selenic acid derivative

$$E - CysSeOH + GSH \rightarrow E\text{-}CysSe - SG + H_2O$$

$$E - CysSe - SG + GSH \rightarrow E - CysSe^- + GSSG + H^+$$

4. Inhibitors of GPx

Irreversibly inhibited by CN^-, unless GSH present

Irreversibly inhibited by iodoacetate

Both GPx & CAT inhibited by $O_2^{\bullet -}$

5. Selenium - Essential for protein synthesis and enzymatic activity of GPx

Animals or cells lose GPx if put on a Se-deficient diet. Increased GPx on selenium addition. Selenite, selenomethionine, and selenocysteine can be used.

Se deficiency signs:

- liver necrosis
- exudative diathesis
- failure to grow and reproduce
- degenerative heart disease (Keshan disease)

Keshan is found in PR China. Low Se in diet. Need 60 μg/day minimum.

Developed countries take in 60-200μg/day.

Low Se areas in Finland and New Zealand do not get Keshan.

Se overdose:

Increased lipid peroxidation and cellular toxicity.

There are Se accumulating plants that poison cattle.

D. Glutathione-S-transferases (GSTs)

Non-Se containing GPx found in 1976 by Lawrence and Burk.

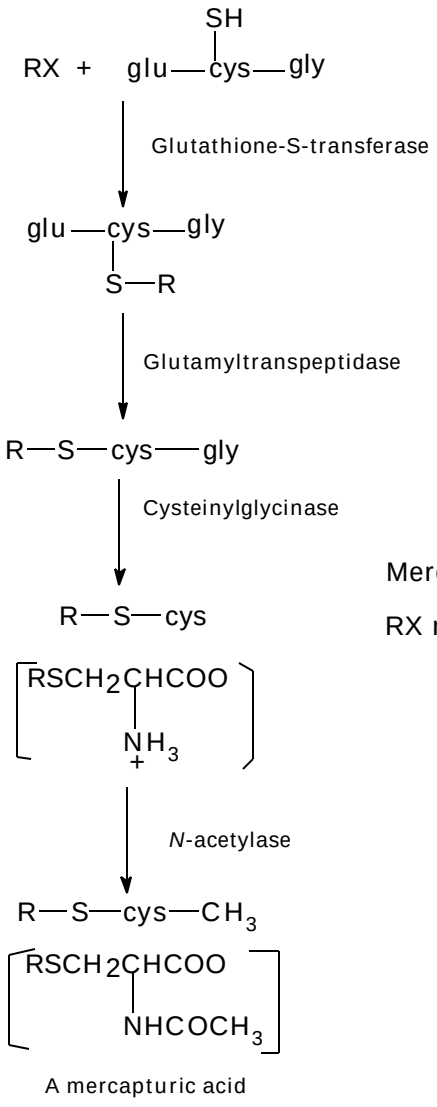
1. Mechanism:

The enzymatic function is the same as glutathione peroxidase, i.e. rids cells of hydroperoxides.

Note: GST does not act on H_2O_2!

$$ROOH + GSH \xrightarrow{GST} GSOH + ROH$$

$$GSOH + GSH \xrightarrow{\text{nonenzymatic?}} GSSG + H_2O$$



A mercapturic acid

(a conjugate of R with N-acetylcysteine)

Mercapturic acid formation.

RX represents the 'foreign' compound.

2. Function:

   a. Biological

      1. GST may function as GPx when Se is low.

      2. Detoxification of foreign cmpds

         - conjugation with GSH (catalytic)

         - binding with ligands which are not substrates

         - covalent bond formation with very reactive compounds leading to inactivation and destruction of GST.

      3. Conjugation reactions involving endogenous compounds, *i.e.,* make steroids, prostaglandins, etc.

3. Location of GST:

   Eukaryotic cells: cytoplasm, nucleus, cell surface, not mitochondria

   Tissue: liver, red cell, intestine

   Accounts for 10% of soluble protein in liver - wow!

Total activity was measured using cumene hydroperoxide as substrate. Results are mostly abstracted from H. Sies *et al*. (1982) *Proc. Third Int. Symp. Oxidases Relat. Redox Systems* (eds. T.E. King *et al.*), Pergamon Press, Oxford, p. 169.

4. Structure of GST

   Liver - dimer with 4 possible subunits

   Ya(22,000 Da); Yb(23,500 Da); Yb′(23,500 Da); Yc(25,000 Da).

   Subunits combine to form 6 isozymes

   YaYa, YaYc, YcYc, YbYb, YbYb′, Yb′b′.

   Only proteins with Ya or Yc exhibit high GPx activity.

   In other organs there are other subunits.

   i.e., placental Yp -correlates with liver cancer

   Yb GST is a major glucocorticoid binding protein

F. Glutathione Reductase (GR)

Function

1. Enzymatic

$$GSSG + NADPH + H^+ \xrightarrow{GR} NADP^+ + 2GSH$$

same assay as GPx, measure NADPH

Other substrates besides GSSG: only mixed disulfides between

GSH & γ-glutamylcysteine or CoA

2. Biological

Removes GSSG, which is toxic

Keeps GSH in reduced form so it can be used.

There are families with low levels of GR in red cells. OK under normal circumstances, but under oxidative stress, red cells hemolyze.

Location in eukayrotic cell: cytoplasm, mitochondria

3. Structure

Human RBC

MW= 104,800 Da

2 identical subunits

The binding positions for the two substrates are of opposite sides of one subunit. Reducing equivalents are transferred from one side of the subunit through the center to the other side. This prevents water from interfering with the catalytic process. Reducing equivalents are transferred through flavin rings (FAD) located at the center and a redox active disulfide bridge adjacent to the flavin.

Need riboflavin in diet to make GR.

G. Glutathione Synthetic Enzymes

1. GSH synthesized by γ-glutamyl cycle

   function of cycle

   - to make GSH

   - transports certain a.a. across membranes - tied to GSH breakdown

   - free radical scavenger

2. Enzymes of γ-glutamyl cycle

   a. γ-glutamylcysteine synthetase

$$\underset{\text{glutamate}}{\text{Glu}} + \text{ATP} \xrightarrow{\gamma\text{-GCS}} \gamma\text{-Glu-}\underset{\text{cysteine}}{\text{CysH}} + \text{ADP} + \text{Pi}$$

   Inhibited by:

   i. *in vivo* by GSH

   ii. L-methionine-S-sulfoximine - also inhibits glutamine synthetase

   iii. Buthionine Sulfoximine (BSO) - does not inhibit glutamine synthetase

   Rat kidney enzyme has a single disulfide bond and two free sulfhydryls per MW 100,000.

   Two subunits (heavy chain 74,000; light chain 24,000).

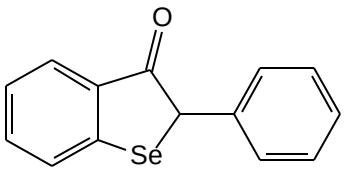
   b. Glutathione Synthetase

$$\gamma\text{-Glu-CysH} + \text{ATP} \xrightarrow{\text{GS}} \text{GSH} + \text{ADP} + \text{Pi}$$

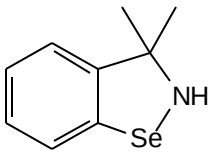
   (GSH = γ-Glu-Cys-Gly)

   Rat kidney GS has MW = 118,000 and 2 identical subunits

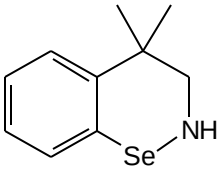
K. Synthetic GPx



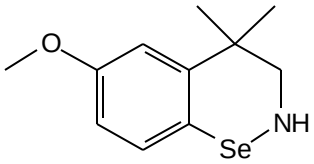
Ebselen



BXT-51056



BXT-51072



BXT-51077

(FRBM, 25:270,1998)

end