Antioxidant Enzymes and Function

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

A. Function

\[
\text{SOD} \quad \text{O}_2^{\bullet-} + \text{O}_2^{\bullet-} + 2 \text{H}^+ \Leftrightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (k_{\text{catalytic}} = 2-4 \times 10^9 \text{ M}^{-1}\text{s}^{-1})
\]

Only enzyme known to act on a radical.
Revolutionary idea: the presence of SOD implies \(\text{O}_2^{\bullet-}\) 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>
<thead>
<tr>
<th>Table 1</th>
<th>Procaryotic Cells - SOD</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>MW/Da</td>
</tr>
<tr>
<td>FeSOD</td>
<td>40,000</td>
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<tr>
<td>MnSOD</td>
<td>40,000</td>
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<table>
<thead>
<tr>
<th>Table 2</th>
<th>Eucaryotic Cells - SOD</th>
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<tbody>
<tr>
<td></td>
<td>MW/Da</td>
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<tr>
<td>MnSOD</td>
<td>88,000</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>32,000</td>
</tr>
<tr>
<td>EC (CuZn) SOD</td>
<td>135,000</td>
</tr>
<tr>
<td>EC MnSOD</td>
<td>150,000</td>
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</tbody>
</table>

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

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

   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)

\[\text{N-C}\]
\[\text{S-H}\]

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
c. **Chemical Modifications of CuZnSOD**

$\text{H}_2\text{O}_2$ - limits activity

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

High concentrations or long exposures cause irreversible inactivation.

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

d. **Catalytic Mechanism** $(E = \text{enzyme})$

\[
\begin{align*}
\text{E-Cu}^{2+} + \text{O}_2{\cdot}^{-} &\rightarrow \text{E-Cu}^{1+} + \text{O}_2 & \text{electron transfer} \\
\text{E-Cu}^{1+} + \text{O}_2{\cdot}^{-} + 2\text{H}^+ &\rightarrow \text{E-Cu}^{2+} + \text{H}_2\text{O}_2 & \text{proton and e}^{-} \text{ transfer}
\end{align*}
\]

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$_2$O$_2$, 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 active site.
  - Cys 107 and Cys 189 forming intrasubunit disulfide bridge found in both proteins.
  - Arg 186 found in both.


  - 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 pl 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 activity decreases.

Mn/FeSOD only slowly inactivated by CN; FeSOD inactivated by H₂O₂.

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 Mn³⁺ and Fe³⁺.

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 H₂O₂, first noted in 1818 that animal tissues could decompose H₂O₂.

Loew in 1901 introduced the name catalase for the natural compound that decomposes H₂O₂.

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

A. Functions:

a. Enzymatic Functions

1. Catalytic

\[
\text{CAT} \quad 2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2 \quad * \text{note oxygenates}
\]

2. Peroxidative first substrate is H₂O₂

\[
\text{ROOH} + \text{HOOH} \rightarrow \text{QO} + \text{ROH} + \text{H}_2\text{O}
\]

A. \( \text{H}_2\text{O}_2 + \text{AH}_2 \rightarrow \text{A} + 2 \text{H}_2\text{O} \)

B. \( \text{ROOH} + \text{AH}_2 \rightarrow \text{H}_2\text{O} + \text{ROH} + \text{A} \)
1. Removes H$_2$O$_2$, adds O$_2$

2. Protects against lipid peroxidation

3. May participate in alcohol metabolism

4. In bacteria, low CAT mutants are hypersensitive to H$_2$O$_2$.

   In Drosophila, 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$_2$O$_2$ 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$_2$O$_2$ 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.

<table>
<thead>
<tr>
<th>Molecular Weights/ Da</th>
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<tbody>
<tr>
<td>Micrococcus lysodeikticus</td>
</tr>
<tr>
<td>Yeast</td>
</tr>
<tr>
<td>Horse liver</td>
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<tr>
<td>Human blood</td>
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</table>

Catalase inhibitor - 3-amino-1, 2, 4- triazole covalently binds His 74, requires $\text{H}_2\text{O}_2$ to inactivate.

![Catalase inhibitor](attachment:image.png)

D. **Catalytic vs. Peroxidative**

Overall: \( \text{ROOH} + \text{HOOH} \rightarrow \text{QO} + \text{ROH} + \text{H}_2\text{O} \)

\( R = \text{H, acyl, aryl} \)

1st step:

\( \text{E-OH}_2 + \text{ROOH} \rightarrow \text{E-O} + \text{ROH} + \text{H}_2\text{O} \)
resting Cmpd I

2nd step:

\[ E-O + HQOH \rightarrow E-H_2O + QO \]

\[ Q = \text{Oxygen} \]

\[ Q = (C = 0) \text{ or } 1-3 \text{ carbon chain peroxidative} \]

\[ k = 10^7 \text{ L mol}^{-1}\text{s}^{-1} \]

\[ k = 10^2 - 10^3 \text{ L mol}^{-1}\text{s}^{-1} \]

Kinetics

\[ \text{CAT Fe(III) + H}_2\text{O}_2 \rightarrow \text{Cmpd I} \]

\[ \text{Cmpd I + H}_2\text{O}_2 \rightarrow \text{CAT(FeIII) + 2H}_2\text{O + O}_2 \]

\[ k_1 = 1.7 \times 10^7 \text{ M}^{-1}\text{s}^{-1} \]

\[ k_2 = 2.6 \times 10^7 \text{ M}^{-1}\text{s}^{-1} \]

Normal kinetics are difficult to do because:

Difficult to saturate CAT with H\(_2\)O\(_2\) due to large k's.

H\(_2\)O\(_2\) 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

\[ \text{moles H}_2\text{O}_2 \text{ used (M s}^{-1}) = 2 k_2 \left[ \text{H}_2\text{O}_2 \right] \left[ \text{Cmpd I} \right] \]

\[ = 2 k_1 \left[ \text{H}_2\text{O}_2 \right] \left[ \text{free catalase} \right] \]

Fix \( \left[ \text{H}_2\text{O}_2 \right] \), then \( [\text{free CAT}] = \frac{\text{moles H}_2\text{O}_2 \text{ used (M s}^{-1})}{2 k_1 \left[ \text{H}_2\text{O}_2 \right]} \)

or \( [\text{CAT}] \propto [\text{H}_2\text{O}_2] \text{ used up} \)

\[ [\text{H}_2\text{O}_2] \text{ exponential, Abs 240 nm, not sensitive} \]
\[ k = \frac{1}{\Delta t} \ln \frac{s_1}{s_2} \]


**Role of NADPH**

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

1. protects CAT from H$_2$O$_2$ inactivation ?
2. source of NADPH for GPx during stress ?


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 complex

- Liposomal / PEG CAT

Pyruvate - from glycolysis

- reacts stochiometrically like catalase, but not catalytically

\[
\begin{align*}
\text{pyruvate} & \quad \text{L(+)Lactic acid} \\
\text{acts as CAT} & \quad \text{binds Mn} \\
\end{align*}
\]

acts as SOD
III. Glutathione and Glutathione Peroxidase - rids of $\text{H}_2\text{O}_2$ or ROOH (hydroperoxide)

A. General Scheme

GSH synthetic enzymes

\[
\begin{align*}
\text{ROOH} & \quad \text{ROH} + \text{H}_2\text{O} \\
\text{GSH peroxidase} & \quad \text{2 GSH} \\
\text{GSSG} & \quad \text{GSSG reductase} \\
\text{NADPH} & \quad \text{NADP} \\
\text{6-P-G} & \quad \text{G-6-P dehydrogenase} \\
\text{stimulates} & \quad \text{inhibits} \\
\end{align*}
\]

GSH = reduced glutathione; \(\gamma\)-Glu-Cys-Gly, \(\gamma\)-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)
B. **Glutathione Peroxidase (GPx)**

Discovered by Mills in 1957

A. Function:

a. **Enzymatic**

\[
\text{ROOH + 2GSH} \rightarrow \text{ROH + H}_2\text{O + GSSG}
\]

Unspecific for hydroperoxides. Can be about anything from \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \):

- Genetic or alimentary deficiency in GPx suffer hemolytic episodes if exposed to drugs generating \( \text{O}_2^* \), \( \text{H}_2\text{O}_2 \), 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.

   Tetrameric protein localized in cytosol.
   Monomer MW = 22,000, 190 amino acids
   Similar substrate specificities as cytosolic GPx (GSHPX-1).
   Both reduce H₂O₂, 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
3. Catalytic Mechanism

SeH \text{ selenol}

SeOH \text{ selenic acid}

\[ E - \text{CysSe}^- + H^+ + \text{ROOH} \rightarrow E - \text{CysSeOH} + \text{ROH} \]

selenol \text{ selenic acid}

\text{derivative}

\[ E - \text{CysSeOH} + \text{GSH} \rightarrow E - \text{CysSe}^- \text{- SG} + \text{H}_2\text{O} \]

\[ E - \text{CysSe}^- \text{- SG} + \text{GSH} \rightarrow E - \text{CysSe}^- + \text{GSSG} + H^+ \]

4. Inhibitors of GPx

Irreversibly inhibited by CN$^-$, unless GSH present

Irreversibly inhibited by iodoacetate

Both GPx & CAT inhibited by O$_2^\cdot$

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$_2$O$_2$!

\[
\text{GST} \quad \text{ROOH} + \text{GSH} \rightarrow \text{GSOH} + \text{ROH}
\]

nonenzymatic?

\[
\text{GSOH} + \text{GSH} \rightarrow \text{GSSG} + \text{H}_2\text{O}
\]

\[
\text{RX} + \text{glu—cys—gly} \xrightarrow{\text{Glutathione-S-transferase}} \text{glu—cys—gly} \xrightarrow{\text{Glutamyltranspeptidase}} \text{R—S—cys} \xrightarrow{\text{Cysteinylglycinase}} \text{R—S—cys—gly} \xrightarrow{\text{N-acetylase}} \text{R—S—cys—CH}_3
\]

A mercapturic acid

(a conjugate of R with N-acetyl(cysteine))

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

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
   \[
   \text{GSSG} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{GR}} \text{NADP}^+ + 2\text{GSH}
   \]
   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 eukaryotic cell: cytoplasm, mitochondria

3. Structure
   Human RBC
   \[
   \text{MW}=104,800 \text{ Da}
   \]
   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
      \[
      \gamma\text{-GCS} \\
      \text{Glu} + \text{ATP} \rightarrow \gamma\text{-Glu-CysH} + \text{ADP} + \text{Pi}
      \]
      glutamate cysteine
      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
      \[
      \gamma\text{-Glu-CysH} + \text{ATP} \rightarrow \text{GSH} + \text{ADP} + \text{Pi}
      \]
      (GSH = γ-Glu-Cys-Gly)
      Rat kidney GS has MW = 118,000 and 2 identical subunits
K. Synthetic GPx

![Ebselen](image1)

![BXT-51056](image2)

![BXT-51072](image3)

![BXT-51077](image4)

(FRBM, 25:270,1998)

end