Investigating post-translational regulation by glutathionylation of glycolytic pathway enzymes of *Saccharomyces cerevisiae*

Vibhu Mishra

MP16019

A dissertation submitted for the fulfilment of MS degree in Science

Indian Institute of Science Education and Research (IISER) Mohali

April 2019

Certificate of Examination

This is to certify that the dissertation titled "**Investigating post translational regulation by glutathionylation of glycolytic pathway enzymes of *Saccharomyces cerevisiae***" submitted by Mr. Vibhu Mishra (MP16019) for thefulfilment of MS degree programme of the Institutehas been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Rajesh Ramachandran **Dr.Shravan Kumar Mishra**

Dr. Anand Kumar Bacchawat

(Supervisor)

April 26 2019

Declaration

I hereby declare that the work presented in this dissertation entitled "**Investigating post translational regulation by glutathionylation of glycolytic pathway enzymes of *Saccharomyces cerevisiae*"**is the result of investigations carried out by me under the guidance of **Prof**. **Anand K. Bachhawat** at the Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Mohali.

This work has not been submitted in part or full for a degree, a diploma, or a fellowship to any other university or institute. In keeping with the general practice of reporting the scientific observations, due acknowledgement has been made wherever the work described is based on the finding of other investigators. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Vibhu Mishra

April 26, 2019

In my capacity as supervisor of the candidate's thesis work, I certify that the above statements by the candidate are true to the best of my knowledge.

Prof. **Anand Kumar Bachhawat**

(Supervisor)

Dedicated to

Friends

Shradha, Shubhi, Saswat, Ashwin & Ashish

Acknolwegement

“for a bowl of water give a goodly meal;

for a kindly greeting bow thou down with zeal;

for a simple penny pay thou back with gold;

if thy life be rescued, life do not withhold”

Narsim Mehta

So, when all things are said and done, we should be thankful and thoughtful to all those who have given us a helping hand in numerous possible ways. I would like to take this opportunity to thank all of them who helped me in all the little or big ways they could.

I would like to thank all my teachers, who have always tried to satisfy my curiosity, because of which I am here. I am thankful to Rajesh sir for all the valuable guidance he has given me. Thanks to Lolitika Madam for teaching developmental biology and for being a friend I am thankful to Shashi sir, Shravan sir, Lolitika , Kuljeet sir for always welcoming me for any help I need. Finally, I feel obliged for my sir , my teacher – Bachhawat sir for all the valuable concerns for my future, for always encouraging me to try once more , I wish I could be a little more little more fruitful and little more worthy. I am obliged for all the freedom and care that he showed towards me and for treating me.

In my lab I am thankful to Shambu Bhaiya for all the help that he provided relating to bacterial transformation, cloning and making deletion mutants and for all the care that he showed towards me relating my work and career.

I am Thankful to Banani Madam for all the time she has given me out of her work. She always helped whenever she can.

Muskan Didi helped me in various problems relating to protein and plasma isolation she would definitely help, if you ask for it or directly say or give some other time

Harsha is bro. this young man helped me in various theoretical and practical issue

Thanks to Swati for all the help we exchanged.

Ashwin is bro too. He always tries to make me happy if he finds me discouraged.

Vidya bhaiya bhaiya, thanks for the help in the lab and outside lab, for all the coffees and samosas.

Finally I would like to thanks Shradha and Shubhi. I will always remember my first time when Shradha helped, me in plasmid isolation. Thanks to all the help in the lab. Prarthna, thank you so much for everything from yeast transformation to protein works and for the other things.

I don't have words to thank Prarthna, for taking all the extra efforts to help me out. I wish I could pay back some day. Thanks to both of you for helping me in my presentation, for playing badminton, sharing lunch and all the good times we had in the lab. I will always be thankful to both of you.

Vibhu Mishra

List of Figures

List of Tables

Contents

Abstract

Glycolysis is the pathway that consumes glucose to produce energy. It is present in all organisms, from bacteria in the deep oceans to complex life forms present on the earth.

During oxidative stress, the cell shifts the glycolytic pathway flux into Pentose Phosphate pathway to generate NADPH to be used by glutaredoxins and thioredoxins to deal with oxidative stress. For this to happen, the flux through the glycolytic pathway has to be shut off by regulation of enzymes of the glycolysis. This has to happen quickly to deal with damaging oxidative stress, since the enzymes' thiols may get permanently oxidised and degraded. So, the cell needs a mechanism that can regulate as well as protect the protein cysteine thiol from permanent oxidation.Glutathionylation is such a mechanism.

Several reports have shown that many glycolytic proteins undergo glutathionylation under cellular oxidative stress. Glutathionylation prevents irreversible oxidation of cysteine residues present in proteins by formation of mixed disulphides. However, the mechanistic role of glutathionylation of these glycolytic proteins has not been well studied.

In the current study, we tried to investigate this process in detail under stress and non-stress conditions.From the literature there are six enzymes known to be glutathionylated in yeast. These enzymes are Glyceraldehyde-3-phosphate dehydrogenase (TDH3), Fructose-bisphosphate aldolase (FBA1), 3-Phosphoglycerate Kinase (PGK1), Enolase (ENO2), Pyruvate Kinase (CDC19) and Triose Phosphate Isomerase (TPI1). To initiate investigations on the project, all six genes were cloned and expressed downstream of a strong yeast promoter. The expression of three of these enzymes Fba1p, Pgk1p, Eno2p could be confirmed. We then evaluated glutathionylation of these enzymes under stressed and non-stressed conditions. To investigate the enzyme involved in the glutathionylation and deglutathionylation process, we examined glutathionylation or deglutathionylation in genetic backgrounds deleted for the different thioredoxins and glutaredoxins that are known to be involved in these pathways. Preliminary results indicate a role of Grx3p in the glutathionylation of Fba1p, Grx1p in the glutathionylation of Pgk1p and Trx2p in the deglutathionylation of Pgk1p. However, more detailed studies are required to confirm these initial findings and determine the mechanisms involved.

Declaration

I hereby declare that the work presented in this dissertation entitled "**Investigating post translational regulation by glutathionylation of glycolytic pathway enzymes of *Saccharomyces cerevisiae*"**is the result of investigations carried out by me under the guidance of **Prof**. **Anand K. Bachhawat** at the Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Mohali.

This work has not been submitted in part or full for a degree, a diploma, or a fellowship to any other university or institute. In keeping with the general practice of reporting the scientific observations, due acknowledgement has been made wherever the work described is based on the finding of other investigators. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Vibhu Mishra

April 26, 2019

In my capacity as supervisor of the candidate's thesis work, I certify that the above statements by the candidate are true to the best of my knowledge.

Prof. **Anand Kumar Bachhawat**

(Supervisor)

Chapter 1: Introduction

1.1Glycolyis

All living organisms need energy to function and to reproduce,which they get through ATP. Though ATP can be produced in different ways, nearly all living organisms can harness ATP through glycolysis, without the need for molecular oxygen (anaerobic).It is surprising to know, no matter how complex or simple the organism is,they all tend to use glycolysis to produce energy.This, in turn shows the simplicity at which a cellular process works no matter what the complexity of life form is.

All organisms perform glycolysis.Glycolysis is an example of living ancient pathway that probably evolved before there was no oxygen in the earth.As organisms evolved to make use of oxygen, they added more efficient pathway to extract more energy from glucose further breaking down pyruvate through tricarboxylic acid cycle to extract more energy [1,2].

The word “Glycolysis” literally means splitting of sugar- glyco- refers to glucose, and -lysis means "splitting".It is the stepwise degradation of glucose and other sugars .Glycolysis occurs in cytosol of the cells

What exactly happens during glycolysis?

During Glycolysis, glucose is broken down to pyruvate through 10 sequential reactions to two molecules of pyruvate. The energy released in breaking those bonds is transferred to carrier molecules, ATP and NADH. Glycolysis can be divided into 2 phases, both of which occur in the cytosol. In first phase, glucose is broken down into two molecules ofGlyceraldehyde-3-Phosphate (G3P) with the consumption of two ATPs.The second phase of glycolysis converts G3P into pyruvate with the release of four ATPs.Glycolysis, therefore, provides the cell with a small amount of energy, and, in aerobic cells, provides the starting materials for the complete oxidation of glucose to carbon dioxide and water [4,5].

The overall reaction of glycolysis can be explained through this chemical equation

$$\mathbf{C_6H_{12}O_6 + 2NAD^+ + 2Pi + 2ADP \rightarrow 2pyruvate + 2NADH + 2ATP}$$

So, for a cell, glucose is a source of energy-it’s its food.It breaks down its food to obtain energy in a sequence of reactions.

Steps of glycolysis

In this section, all steps of glycolysis with their general regulation are mentioned. Later,we have dealt with regulation in specificity to the irreversible steps of glycolysis.

There are 10 sequential steps of glycolysis,which in the end generates pyruvate and ATP.

1. **Phosphorylation of glucose**

The first step in glycolysis is the transport of glucose across the cell membrane by members of the hexose transporter family [5,6].Subsequently, intracellular glucose is phosphorylated to glucose-6-phosphate. In the yeast *S. cerevisiae*, there are three isozymes that phosphorylate glucose: glucokinase (encoded byGLK1**),** hexokinase I (encoded by HXK1*),* and hexokinase II (encoded by HXK2). These isozymes have different affinities for glucose and ATP and different specificities toward other sugars, such as fructose and mannose.

Furthermore, the transcriptional regulation of the genes are different, depending on the source and the amount of carbon [7].In contrast to hexokinases from other organisms, *S. cerevisiae* hexose kinases are not inhibited by their product glucose-6-phosphate[8].The flux of Glucose-6-phosphate can be directed at this point into the pentose phosphate pathway also .

2. **Isomerisation of Glucose-6-phosphate to Fructose-6-phosphate**

Glucose-6-phosphate(G6P) is then rearranged into fructose 6-phosphate (F6P) by glucose phosphate isomerase. Fructose& mannose can also enter the glycolytic pathway by phosphorylation at this point.Phosphoglucose isomerase isomerises the G6P to F6P.This reaction is freely reversible under normal cell conditions. However, it is often driven forward because of a low concentration of F6P, which is constantly consumed during the next step of glycolysis. Under conditions of high F6P concentration, this reaction readily runs in reverse[4].Yeast has single glucose-6-phosphate isomerase -PGI .D-arabinonamide-5-phosphate and D-arabinohydrazine-5-phosphate are known to inhibit PGI1 in yeast[9].

3. Phosphorylation of Fructose 1,6-bisphosphate

The enzyme phosphofructokinase (PFK) uses another ATP molecule to transfer a phosphate group to fructose 6-phosphate to form fructose 1, 6-bisphosphate.Yeast has PFK1 and PFK2 which together form hetero-octamer.It is the first irreversible step in glycolysis and most significant one.

4. Cleavage of Fructose 1,6-bisphosphate

The enzyme Aldolase splits fructose 1,6-bisphosphate into two sugars isomers - dihydroxyacetone phosphate(DHAP) and glyceraldehyde phosphate(G3P).There are two classes of aldolases, which have different catalytic mechanisms: class I enzymes are found in animals and the class II enzymes are produced in bacteria and fungi .Class I enzymes do not require a metal ion, forms a Schiff base intermediate between an active site lysine and a substrate carbonyl group while class II enzymes require an active-site divalent metal ion.

Yeast has single aldolase Fructose-bisphosphate aldolase -FBA1[116].The reaction is reversible and enzyme takes part in both glycolysis and gluconeogenesis[4].

5. Isomerization of G3P and DHAP

Triose phosphate isomerase (TIM) catalyses the reversible interconversion of G3P and DHAP. Only G3P can be used in glycolysis, therefore TIM is essential for energy production, allowing two molecules of G3P to be produced for every glucose molecule, thereby doubling the energy yield.PEP functions as a competitive TPI inhibitor, PEP directly binds into the catalytic pocket of TPI .In humans, the PYK–TPI feedback loop, leading to the regulation of glycolysis and the PPP to adapt to oxidative stress conditions, is the consequence of active-site competitive TPI inhibition by the PK substrate PEP [10].

Yeast has single Triose phosphate isomerase TPI1.Tpi expression in plants has been linked to methylglyoxal toxicity.Increases in MG leads to the increase in TPI which results in decrease of DHAP and consequently decrease in the level of toxic MG [11]. Iron has a role in determining Tpi mRNA half-life and its transcription is controlled by activators Reb1p, Gcr1p, and Rap1p through binding sites in the 5' non-coding region .Yeast Tpi1p also shows inhibition of activity by PEP (phosphoenolpyruvate) which stimulates redox metabolism in respiring cells[12,13].

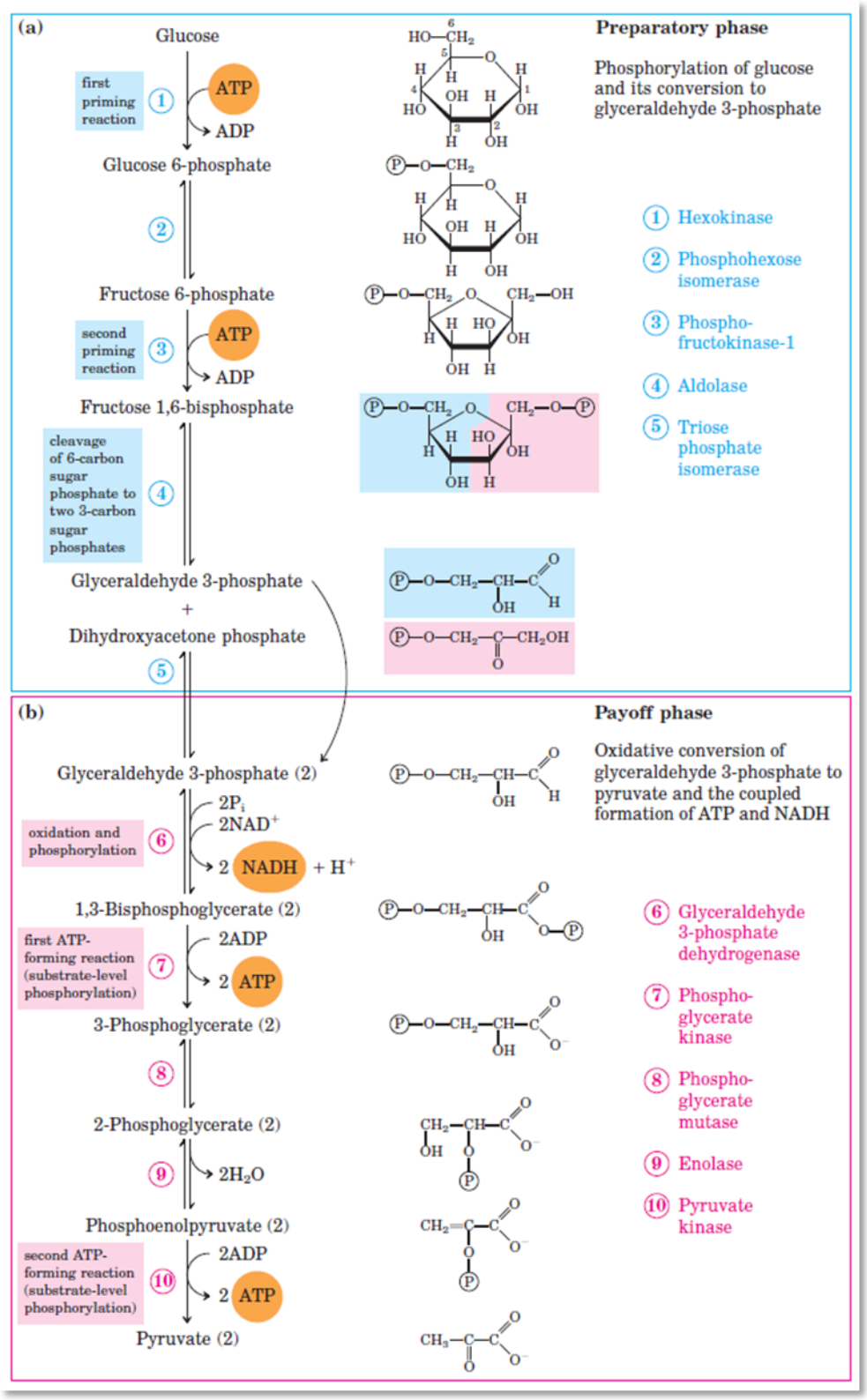


Figure1.1 Glycolysis pathway in general .Glucose undergoes ten sequential steps of degradation to finally generate pyruvate and ATP

(from https://laboratoryinfo.com/glycolysis-steps-diagram-energy-yield-and-significance[14])

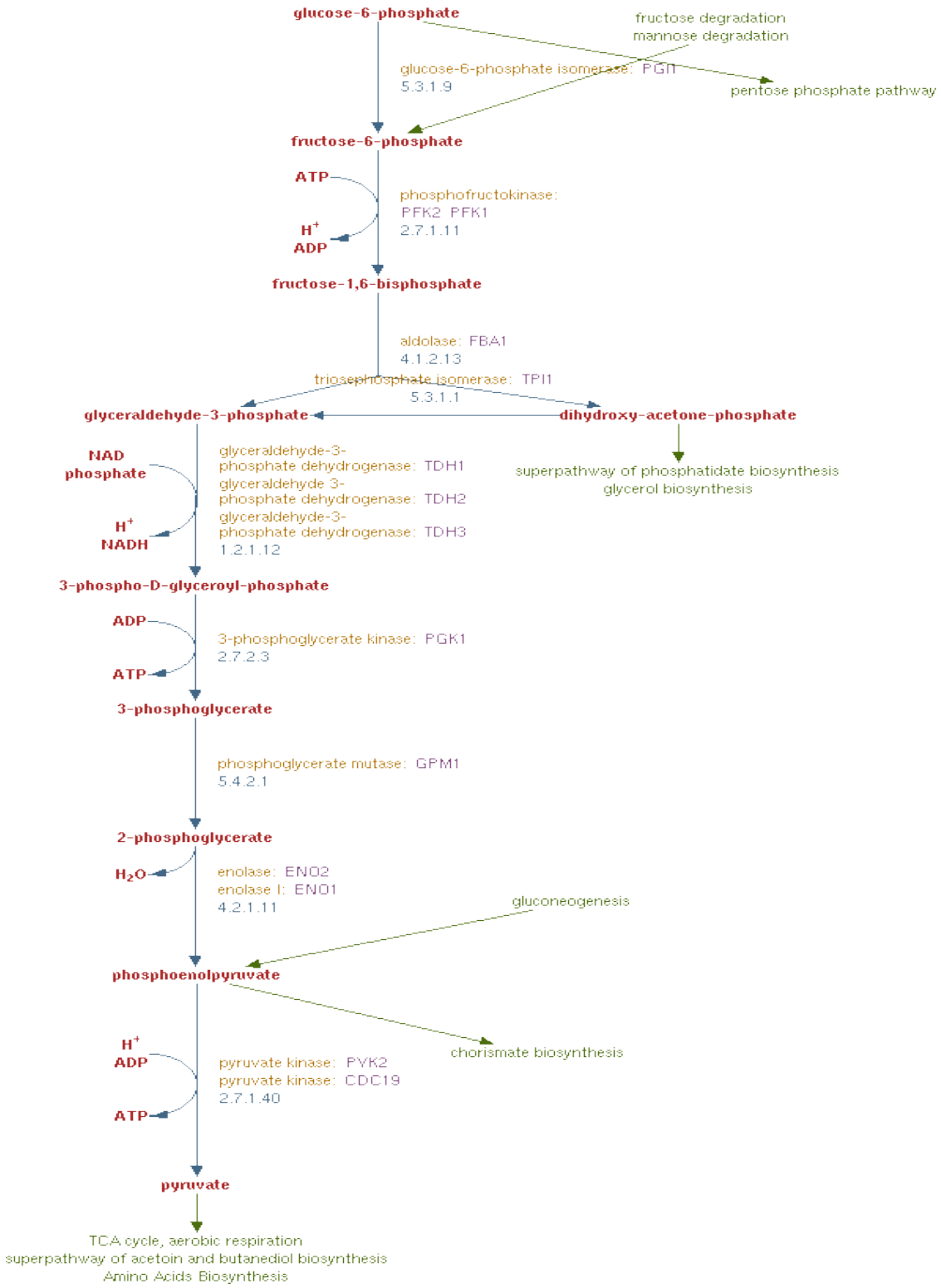


Figure1.2 Glycolytic pathway in yeast .The various isozymes catalysing the steps are mentioned along with their enzyme classes.Glycolytic enzymes feed into a number of other catabolic pathways and in turn being contributed by various substrates along the way.It is one of central metabolic pathway in yeast[15]

(From SGD pathways database[15])

6. Conversion of Glyceraldehyde-3-phosphate (G3P) to1,3-Bisphosphoglycerate (1,3BPG)

Glyceraldehyde-3-phosphate dehydrogenase(GAPDH) reversibly catalyses the oxidation and phosphorylation of G3P to the energy-rich intermediate 1,3BPG. NAD^+ is a co-substrate for this reaction.

GAPDH displays diverse non-glycolytic functions depending upon its subcellular location.There are three genes for GAPDH in yeast -TDH1, TDH2 and TDH3.TDH3 and TDH2 are known to have different functions during oxidative stress[6].Interestingly, these catalytically active enzymes are found in the cytoplasm and cell wall. Tdh2p and Tdh3p are detected in exponentially growing cells whereas Tdh1p is primarily detected during stationary phase [16].They are differentially regulated by oxidative stress.

7. Conversion of 1,3BPG to 3PG

Phosphoglycerate kinase (PGK) reversibly catalyses transfer of high-energy phosphoryl groups from the acyl phosphate of 1,3-bisphosphoglycerate to ADP to produce ATP. The ATP is made by substrate-level phosphorylation, where a phosphate group is transferred from 1,3BPG directly to ADP [17].

Yeast has one PGK1 whose transcription is activated by the transcription factors Rap1p, Abf1p, and Reb1p, each of which bind to sequences in the PGK1 promoter [18].Recent research in frogs which can withstand freezing temperatures indicates that PGK is up regulated by the cold and more specifically by low levels of oxygen. PGK can also function to cleave disulfide bonds. PGK has been shown to cleave disulfide bonds in the protein zymogen plasmin to produce the active form of the protein [19,20].

8. Conversion of 3-Phosphoglycerate(3PG) to 2-Phosphoglycerate (2PG)

Phosphoglycerate mutase (PGAM) catalyses the transfer of the phospho group from the C3 position to the C2 position, in preparation for next step of ATP synthesis. PGAM enzymes from different sources have different reaction mechanisms. For instance, some PGAM enzymes (vertebrates, fungi, certain bacteria) use 2,3-bisphophoglycerate as a cofactor to phosphorylate a serine residue for the reaction, whereas other PGAM enzymes (plants, certain invertebrates, algae, certain bacteria) carry out intramolecular phosphoryl group transfer via an active site residue without the need of a cofactor[17].

9. Conversion of 2-phosphoglycerate to phosphoenolpyruvate

Enolase (phosphopyruvate hydratase) is an essential glycolytic enzyme that catalyses the reversible dehydration of 2-phosphoglycerate to the high-energy intermediate phosphoenolpyruvate. Fluoride ions strongly inhibit Enolase, which forms a fluorophosphate complex with magnesium at the active site.

ENO1 and ENO2 are the two *S. cerevisiae* genes encoding phosphopyruvate hydratase which take part both in glycolyis and gluconeogenesis.Glucose represses Enolase I and simultaneously induce Enolase II. Enolase catalyses the first common step of glycolysis and gluconeogenesis. Gluconeogenic enolase I shows substrate inhibition for 2-phosphoglycerate (glycolytic substrate) and glycolytic enolase II is substrate-inhibited by phosphoenolpyruvate (gluconeogenic substrate). Normal concentrarions of fructose 1,6 bisphosphate in the body is known to inhibit the gluconeogenic reaction[22].Enolase is also transcriptionally controlled. ENO1 expression is induced more than 20-fold in cells grown on glucose as the carbon source than ENO1.There are cis-acting elements present upstream of start site that are responsbile for differential expression of Eno1p and Eno2p protein [23].

10. Conversion of Phosphoenolpyruvate to Pyruvate

Pyruvate kinase (PK) catalyses the final step in glycolysis, the conversion of PEP to pyruvate with the transfer of the high-energy phosphate group from PEP to ADP, thereby generating ATP. PK requires both Mg^{2+} and $K^{+}$for activity.

PK helps control the rate of glycolysis, along with phosphofructokinase and hexokinase. The pyruvate may have different fates.Under aerobic conditions, enters the TCA cycle and is further broken down to produce more ATP through oxidative phosphorylation. Alternatively, pyruvate can be anaerobically reduced to lactate in cells lacking mitochondria, or under hypoxic conditions, such as those found in hard working muscle tissues. anaerobic breakdown of pyruvate in yeast produces ethanol during fermentation [17].Yeast contains two pyruvate kinase- CDC19 and PYK2. CDC19 is tightly regulated and activated by fructose-1,6-bisphosphate (FBP) whereas PYK2 is subject to glucose repression and appears to be insensitive to FBP levels, suggesting that it may be active when FBP levels are too low to activate CDC19 (131). Therefore, PYK1 appears to be the main pyruvate kinase in the glycolytic pathway (131,132,133).

1.2Glycolyis and Oxidative stress

Multiple enzymes of the glycolytic pathway get inhibited including glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase M2, and phosphofructokinase-1.In yeast,exposure to H_2O_2 led to inhibition of GAPDH,enolase and alcohol dehydrogenase[27].Briefly, glycolysis inhibition promotes flux into the pentose phosphate pathway to generate NADPH. NADPH is important in the sense that, it provides the reducing power that assists the antioxidant systems in manitainence and recycles oxidized glutathione [86,87].So glycolytic enzyme inhibition helps the cell to focus on dealing with oxidative stress rather than investing in continuous production of energy.

The pentose phosphate pathway (PPP) provides ribose 5-phosphate for nucleotide synthesis and NADPH as a cofactor for fatty acid synthesis.Also,NADPH helps in the glutaredox and thioredoxin system to regain reductive power. These metabolites are needed for cell to proliferate and for this reason flux in the PPP is thought to be increased in cancer[28].External or internal disturbance with the cellular oxidative stress are known to sensitively control glycolysis and the Warburg effect in hepatoma cells[28].

1.3 The Pentose Phosphate Pathway and NADPH Production

As the glucose is imported inside the cells through hexose transporters, Hexokinases (HK) phosphorylate glucose to produce glucose-6-phosphate(G6P). Glucose phosphorylation creates a concentration gradient to draw more glucose in. G6P lies at connecting the link of glycolysis, glycogen synthesis—(through formation of glucose-1-phosphate) and the oxidative arm of the pentose phosphate pathway (ox-PPP). The fate of G6P is determined by cell type and demands of the cell. The ox-PPP is imperative producer of cellular NADPH and is thus important for antioxidant defense [28]. Only the oxidative arm of PPP generates NADPH during the formation of ribulose-5-phosphate [28].First NADPH is generated during the formation of 6 phosphogluconate by glucose6-phosphate dehydrogenase(G6PD).In the next step,6 phosphogluconate lactone ring is opened by phoshopgluconolactonase to form 6 phosphogluconate, which is then further decarboxylated by 6-phosphogluconate to finally generate one more NADPH and ribulose-5-phosphate.The net yield per unit of G6P is therefore two NADPH and ribulose-5-phosphate.Ribulose-5-phosphategenerates ribose-5-phosphate in the non- oxidative part of PPP. The G6P carbon may be recycled back into glycolysis as the PPP enzyme transketolase and transaldolase recycles the G6P the

non-oxidative branch of PPPby forming the glycolytic intermediates glyceraldehyde-3-phosphate (G3P) and fructose-6-phosphate (F6P) [28]. G6PD is known to be regulated by phosphorylation, protein–protein interaction, and by translocation to the plasma membrane upon growth factor stimulation [28]. G6PD responds to the NADP+/NADPH ratio being allosterically activated by the NADP+ and inhibited by NADPH [28]. As, NADPH is utilised by Antioxidant enzymes to reduce ROS-induced damage, NADP+ levels increase, which in turn stimulates the activity of the ox-PPP to produce more NADPH and maintain cellular reducing power.Thus,the diversion of glycolytic flux into the ox-PPP pathway plays a vital role in antioxidant defense .

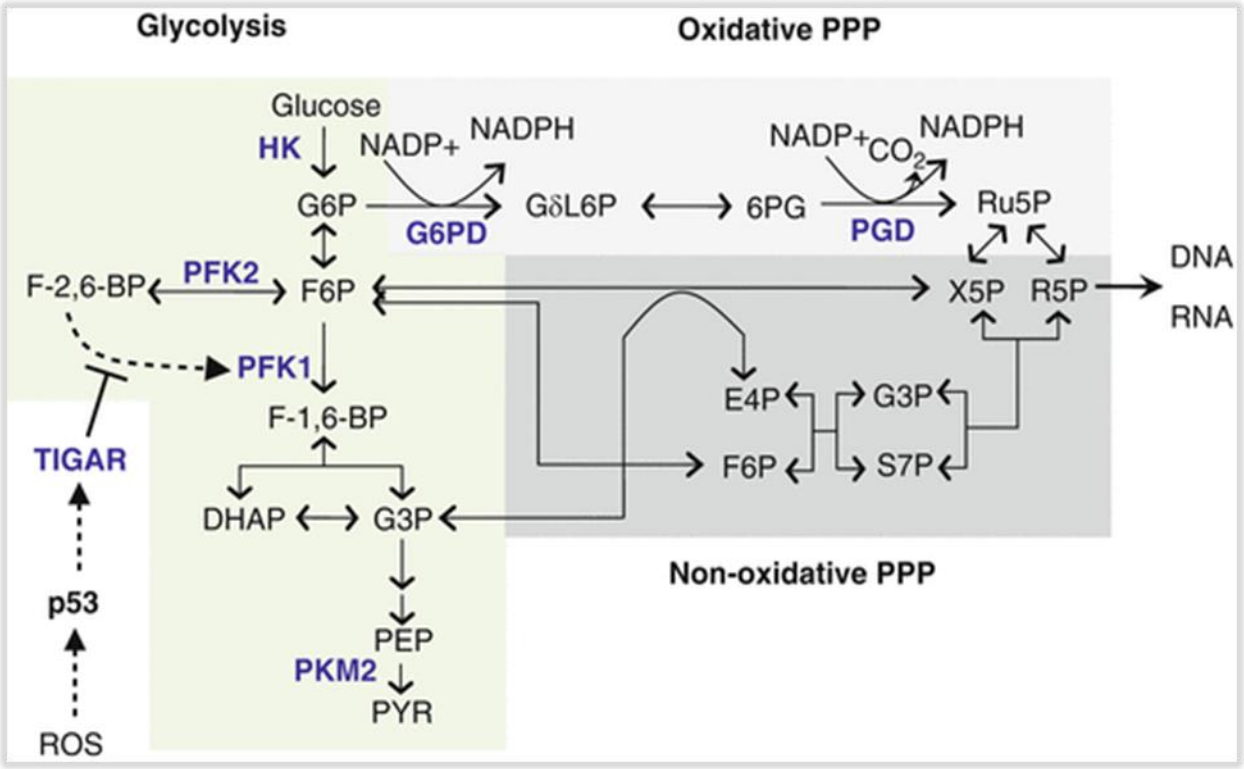


Figure1.3 Glycolysis and the pentose phosphate pathway (PPP)

The PPP is composed of two distinct arms, the oxidative branch (*light blue*) and the non-oxidative branch (*gray*). While both arms produce ribose-5-phosphate, a precursor for nucleotide synthesis, only the oxidative branch concomitantly produces NADPH. Glycolytic flux enters the oxidative branch via glucose-6-phosphate dehydrogenase (G6PD). Fructose-2,6-bisphosphate (F-2,6-BP) activates phosphofructokinase-1 (PFK1) to promote glycolysis (*light green*). In response to reactive oxygen species (ROS) and UV stress, p53 activates TIGAR (TP53-induced glycolysis and apoptosis regulator). TIGAR degrades F-2,6-BP, thereby inhibiting PFK1. This allows glycolytic flux to be diverted into the oxidative arm and enhances NADPH production to fuel the cellular antioxidant systems. Metabolic enzymes are shown in dark blue.

(From Book chapter Innovative Medicine: Basic Research and Development: Diverting Glycolysis to Combat Oxidative Stress Edouard Mullarky and Lewis C. Cantley[28])

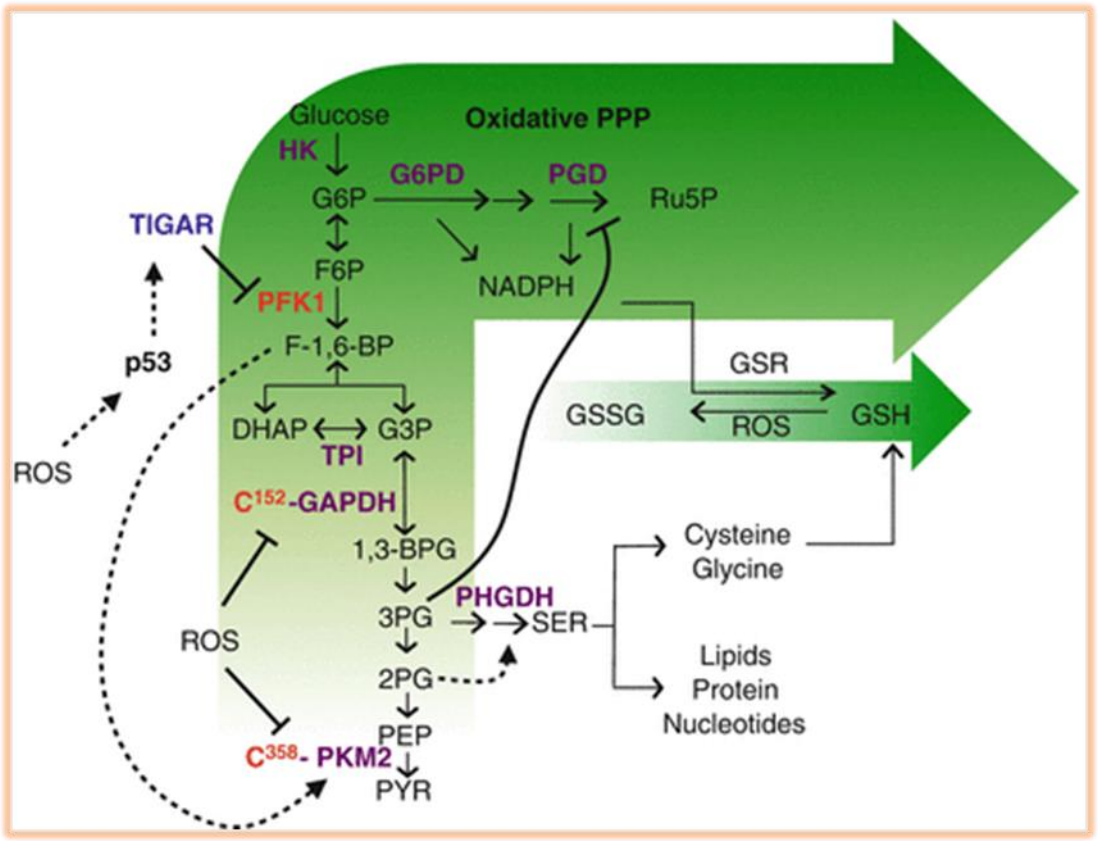


Figure1.4 Reactive oxygen species (ROS)-mediated inhibition of glycolysis reroutes flux into the oxidative arm of the pentose phosphate pathway.

ROS inactivates glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the pyruvate kinase isoform PKM2 by directly targeting cysteine residues. Alternatively, ROS and UV stress can trigger p53-dependent TIGAR (TP53-induced glycolysis and apoptosis regulator) activation that inhibits phosphofructokinase-1 (PFK1). Glycolytic inhibition promotes flux into the oxidative pentose phosphate pathway to produce NADPH and fuel cellular antioxidant systems *(*graded green arrow). For example, NADPH is consumed by glutathione reductase (GSR) to recycle oxidized glutathione (GSSG). PKM2 inhibition is unique in that it allows for a diversion of flux into the serine synthesis pathway. Serine not only contributes to the synthesis of macromolecules but is also a precursor for glutathione (GSH). Serine synthesis is activated by a buildup of 2-phosphoglycerate (2PG), which prevents 3-phosphoglycerate (3PG)-induced inhibition of the oxidative pentose phosphate arm. Enzymes are shown in *purple*. ROS targets are shown in red [28]

(From book chapter -Innovative Medicine: Basic Research and Development: Diverting Glycolysis to Combat Oxidative Stress Edouard Mullarky and Lewis C. Cantley)

1.4 Regulation of glycolysis

The flux through the glycolytic pathway changes in response to both intrinsic and extrinsic factors. The rate of conversion of glucose into pyruvate is regulated to meet two major cellular needs: (1) the production of ATP and (2) the requirement of building blocks for synthetic reactions, such as the formation of fatty acids. In glycolysis, the reactions catalyzed by hexokinase, phosphofructokinase, and pyruvate kinase are irreversible as they involve large negative ΔG. Hence, these enzymes would be expected to have regulatory as well as catalytic roles. In fact, each of them serves as a control site. The enzyme activities are regulated by the reversible binding of allosteric effectors or by covalent modification. In addition, there are controls at the level of transcription level also to meet the changing metabolic needs.The time required for reversible allosteric control, regulation by phosphorylation, and transcriptional control is usually in milliseconds, seconds, and hours, respectively[4].

Trancriptional regulation

In yeast, glucose activates the transcription of Hxk genes.Glucose induction of certain glucose carrier genes are Hxk2.cAMP activates protein kinase A(PKA) which in turn acitvates enzymes in glycolysis and inhibits enzymes in gluconeogenesis.PKA also causes induction of growth-related genes and repression of stress-related genes.cAMP , in turn gets activated by combined efforts of Gpr1(activated by high glucose levels) and Phosphorylated glucose inactivating Gα protein homologue Gpa2 .Gpa2 activates adenylate cyclase (Cyr1)[29].

In case of yeast, Glucose repression is a mechanism that adapts yeast cells for the fermentation of glucose, the preferred carbon source [29], by repressing a large number of genes at the level of transcription. Transcription ofSUC2 (encoding invertase), GALgenes (encoding proteins involved in galactose metabolism), MAL genes (encoding proteins involved in maltose metabolism), HXK1(encoding hexokinase I), and genes encoding enzymes of the glyoxylate shunt, the tricarboxylic acid (TCA) cycle, and gluconeogenesis are all repressed in the presence of glucose. In addition, genes involved in respiration and other mitochondrial activities are also repressed by glucose. In *S. cerevisiae*, glucose repression leads to the occurrence of diauxic growth on glucose.Hxk1 deletions showed derepression.Thus,a central role of Hkx1p in repression of glucose is there[29].

Hexokinase Regulation

Two isozymes - hexokinase-1 and hexokinase-2, can phosphorylate keto- and aldohexoses in yeast, whereas a third isoenzyme, GLK, is specific for aldohexoses.

Hexokinase, the enzyme catalyzing the first step of glycolysis, is inhibited by its product, glucose 6-phosphate.High concentration of glucose 6-phosphate signals that the cell no longer require glucose for energy, for storage as glycogen or as a source of biosynthetic precursors and glucose will be left unused.Various factors may affect the inhibition for e.g. the concentration of ATP, Mg^{2+},in case of heoxkinase of brain of guinea pig,ADP also inhibited the the enzyme activity[30].

In hexokinase reaction in yeast,AMP acts as a competitive inhibitor for ATP and a true non-competitive inhibitor for glucose[30].Another well characterized allosteric regulator of the glycolytic flux is trehalose 6-phosphate, which acts as a feedback inhibitor of hexokinase , thereby preventing the hyper-accumulation of sugar phosphates.Glucokinase was unaffected[31].

Glucokinase has a much higher Km for glucose and is not product inhibited.In mammals it is found in liver and becomes important when the levels of glucose are very high.[31].

Phosphofructokinase Regulation

Phosphofructokinase (PFK) is key regulatory enzyme of glycolysis. It catalyses an irreversible step.PFK switches between two conformations, which are known as tense (T) and relaxed (R). When the enzyme is in the R state, it has a high affinity for its substrate, fructose-6-phosphate (F6P), and thus a high activity level[32]. PFK's activity is inhibited by allosteric regulation by ATP itself.ATP binds to allosteric site in PFK and changes the conformation to a T state.[32]

ATP binds in two places on PFK: in the active site as a substrate and in the regulatory site as a negative modulator. ATP bound in the regulatory site acts as a modulator by lowering the affinity of PFK for its other substrate, fructose-6-phosphate[35].PFK is also inhibited by abundant cellular concentrations of citrate, another marker of a high energy state of a cell. When citrate levels are high, the cell can obtain more than enough energy from the citric acid cycle and does not need glycolysis to supply more carbons into the citric acid cycle[34].

Phosphoenolpyruvate one of last products of glycolysis also allosterically inhibits PFK[33].

PFK activity is also regulated by pH activity .The affinity of the inhibitory site(s) of phosphofructokinase for ATP is affected by hydrogen ion concentration and that aging causes a change of conformation of the enzyme, thus blocking the control mechanism of phosphofructokinase[36]. A low pH also decreases the affinity of the enzyme for its substrate Fructose 6-phosphate as observed in the skeletal muscles of frog[36].

When cellular energy is low, glycolysis should be upregulated. PFK is allosterically activated by high levels of AMP & ADP[37,38,40].The activity of phosphofructokinase is influenced by a variety of ligands. For the regulation of cellular metabolism the activation of the enzyme by AMP and fructose-2,6-bisphosphate is considered most important.

The effect of AMP contributes to the homeostasis of ATP , while Fru-2,6-P2 is involved in the coordination of glycolysis and gluconeogenesis[37,38,39].PFK from yeast is effectively activated by AMP and fructose-2,6-bisphosphate by increasing the affinity of the enzyme to fructose-6-phosphate and the maximum activity toward this substrate. The enzyme is activated by AMP and fructose-2,6-bisphosphate both at high and at low concentrations of ATP. The fructose-6-phosphate affinity is more enhanced by fructose-2,6-bisphosphate than by AMP[37,38].

Another allosteric activator of PFK is fructose 2, 6 bisphosphate. F-2,6-BP is not an intermediate in the glycolytic pathway. F-2,6-BP also overcomes the inhibitory effect of ATP[37,38,39].Infact,Fructose2,6 bisphosphate regulates both glycolysis and gluconeogenesis.

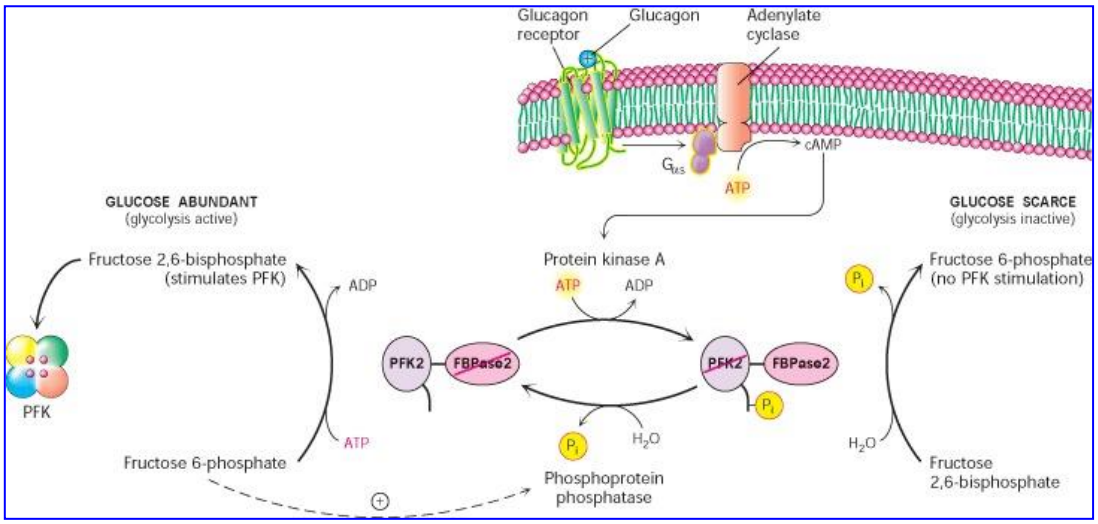


Figure1.5 Control of the Synthesis and Degradation of Fructose 2,6-Bisphosphate.A low blood-glucose level as signalled by glucagon leads to the phosphorylation of the bifunctional enzyme and hence to a lower level of fructose 2,6-bisphosphate, slowing glycolysis. High levels of fructose 6-phosphate accelerate the formation of fructose 2,6-bisphosphate by facilitating the dephosphorylation of the bifunctional enzyme[4].

Some of the protein substrates of PKA have been identified. These include, in particular, enzymes of carbon and lipid metabolism: trehalase, glycogen synthase, glycogen phosphorylase, phosphofructokinase 2, fructose- 1,6- bisphosphatase, isocitrate lyase, phosphatidylserine synthase and phosphatidate phosphatase[41].

Pyruvate kinase Regulation

The yeast protein kinase Mck1,a member of the glycogen synthase kinase 3 family of protein kinases , negatively regulates Pyk1/Cdc19 activity, possibly by direct phosphorylation, and suggest that many of the phenotypes of mck1D mutants may be explained by the hyperactivity of Pyk1[42].

Later, Tpk1p, a cAMP-dependent protein kinase catalytic subunit, phosphorylates purified pyruvate kinase in vitro, and that loss of the cAMP-dependent protein kinase regulatory subunit, Bcy1p, increases pyruvate kinase activity in vivo. We find that purified Mck1p inhibits purified Tpk1p (catalytic subunit of PKA) in vitro, in the presence or absence of Bcy1p(regulatory subunit)[138].PKA can phosphorylate yeast Pyk1p in vitro in the presence of cAMP and cGMPand mainly Tpk2 catalytic subunit was involved[43].

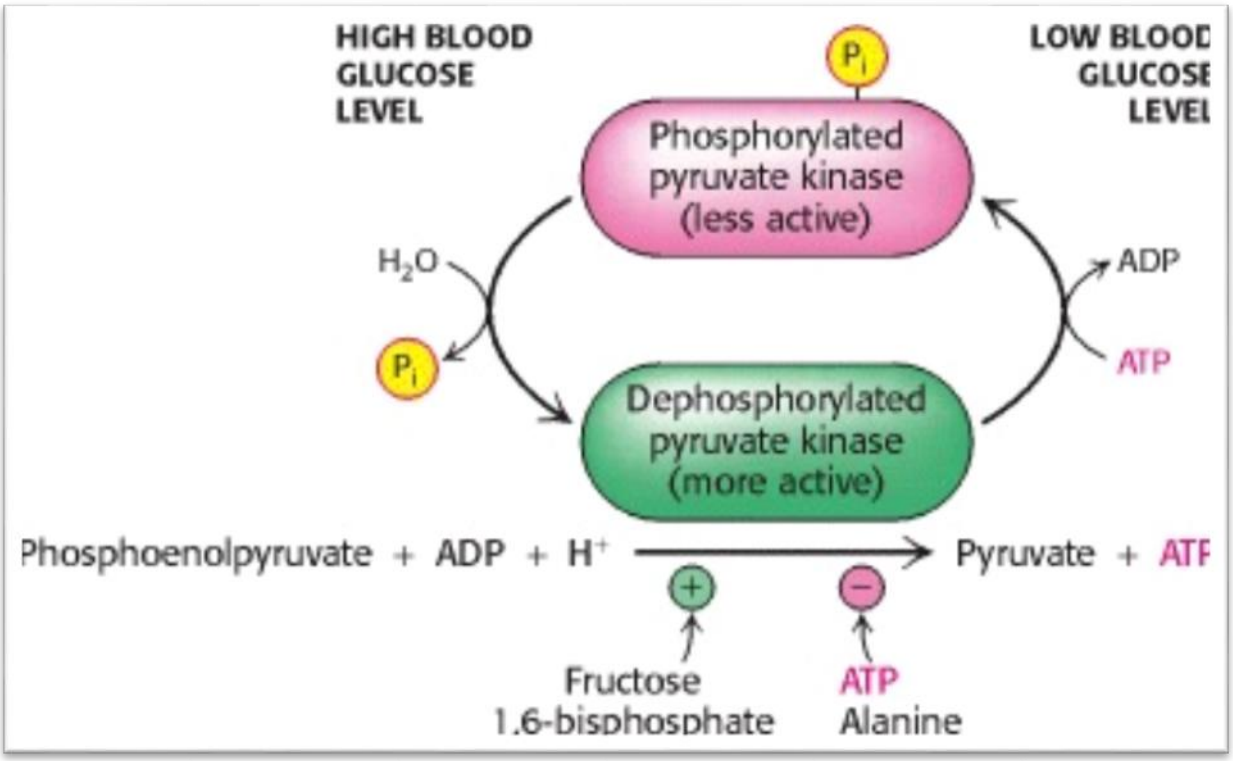


Figure1.6 Control of the Catalytic Activity of Pyruvate KinasePyruvate kinase is regulated by allosteric effectors and covalent modification.ATP ,Alanine negatively regulate PK activity while Fructose1,6-bisphosphate is a positive effector[4]

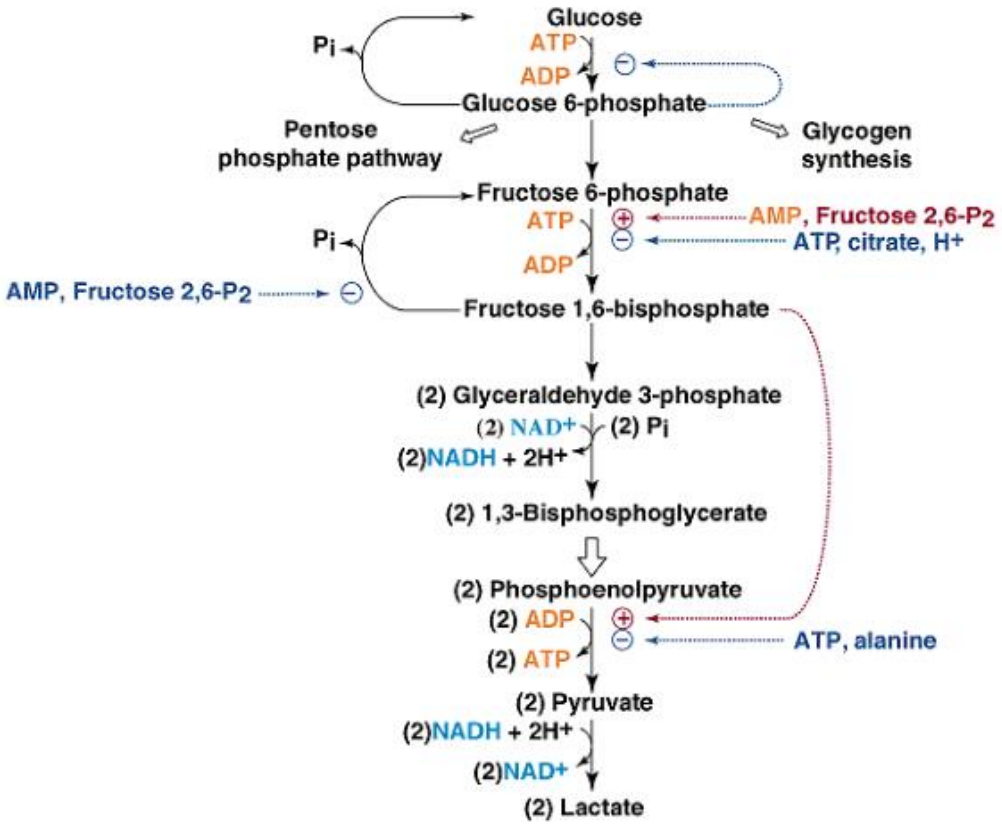


Figure1.7 Regulation of glycolysis(44)

(from http://imed.stanford.edu/curriculum/session6/content/09-)

Regulation through Post-translational modifications

Post translational modifications(PTMs) modulate protein activity very fast within seconds, PTMs may create a cascade of responses ,modifying a large number of proteins at the same time against a common stimulus (for instance a specific kinase phosphorylating a set of metabolic enzymes).Several metabolic enzymes are exposed to multiple post-translational modifications, suggesting that yeast cells can use different PTMs and/or combinations of them to specifically respond to environmental changes[45]. Phosphorylation and lysine modifications (ubiquitination and acetylation) are discussed here.

Phosphorylation is the best studied and more frequent reversible post-translational modification in yeast. A combined proteomic strategy was applied,to identify in vivo functionality of enzyme phosphorylation under different growth conditions, to relate phosphorylation status with physiological enzyme activity [45].The phosphorylation status of the various metabolic enzymes together with the phosphorylating enzymes would greatly

improve our understading of metabolic pathway control by phosphorylation. According to phospho-proteomic data, a large number of metabolic enzymes are phosphorylated, on one or more sites [45].

Interestingly, the main target of phosphorylation is Pfk26, one of the two isoforms of 6-phosphofructo-2-kinase, responsible for the synthesis of the allosteric regulator fructose-2,6-bisphosphate. Also Pfk1 and Pfk2 and Cdc19, catalyzing the two irreversible steps of the glycolytic pathway, appear to be phosphorylation hubs, thus showing that these steps of glycolysis are the most regulated by phosphorylation.

The cyclin-dependent kinase Pho85 is mainly responsible for these phosphorylations. Pho85 is a multifunctional kinase, which regulates cell-cycle progression, cell polarity and the actin cytoskeleton, gene expression, metabolism and the signaling of environmental changes [45]. Pho85 activity depends on the association with cyclin regulatory subunits, which confer substrate specificity and targets the enzyme to different cellular functions [45]. The role of Pho85-dependent phosphorylation on some of these enzymes has been functionally characterized.

It phosphorylates proteins catalyzing key enzymatic steps, such as both isoforms of pyruvate kinase, Cdc19 and Pyk2 [45]. PKA phosphorylation on Ser22 was shown to stimulate Cdc19 activity, although cells expressing Cdc19-S22A are still able to grow in glucose [45]. PKA also phosphorylates the 6-phosphofructo-2-kinase Pfk26 on Ser644, increasing its enzymatic activity [45]as well as on some other residues, whose physiological relevance has not been fully understood [45].

Ubiquitination and acetylation

Another system to regulate metabolic pathways under specific growth conditions is by modulating enzyme stability, which is achieved through the ubiquitin/proteasome pathway.

Lysine residues can also be modified by acetylation. Lysine acetyl-transferases (KATs) or a histoneacetyltransferases (HATs) can acetylate lysine residues on their ε-amino groups.lysine deacetylases (KDACs) or histone deacetylases (HDACs) reverse the process[40]. Acetylation depends on the concentration of acetyl-CoA, a central metabolite.

The two pathways with the highest concentration of modified lysines are glycolysis (Pgi1, Pfk26, Pfk1, Pfk2, Fba1, Tpi1, Pgk1, Gpm1, Eno1, Eno2 and Cdc19) and fermentation (Pdc1,

Adh1, Ald4 and Ald6). Most of these enzymes are irreversible.It is also excitingto notice that most of these enzymes were found to under go either ubiquitinated or acetylated, suggesting that acetylation and ubiquitination can be mutually exclusive post-translational modifications[45].

1.5Oxidative Stress & Redox modifications of proteins

Oxidative stress is a situation in a cell in which cellular redox balance is disturbed because of excessive production of reactive oxygen/nitrogen species (ROS/RNS) and/or damage of cellular antioxidant defenses [49]. ROS/RNS can cause specific, reversible and/or irreversible oxidative modification on redox sensitive proteins that may affect the activity or function of the oxidized protein or cause permanent damage [50]. Most protein oxidation products are commonly considered as cellular indicators of oxidative/nitrosative stress/damage [49,50] .

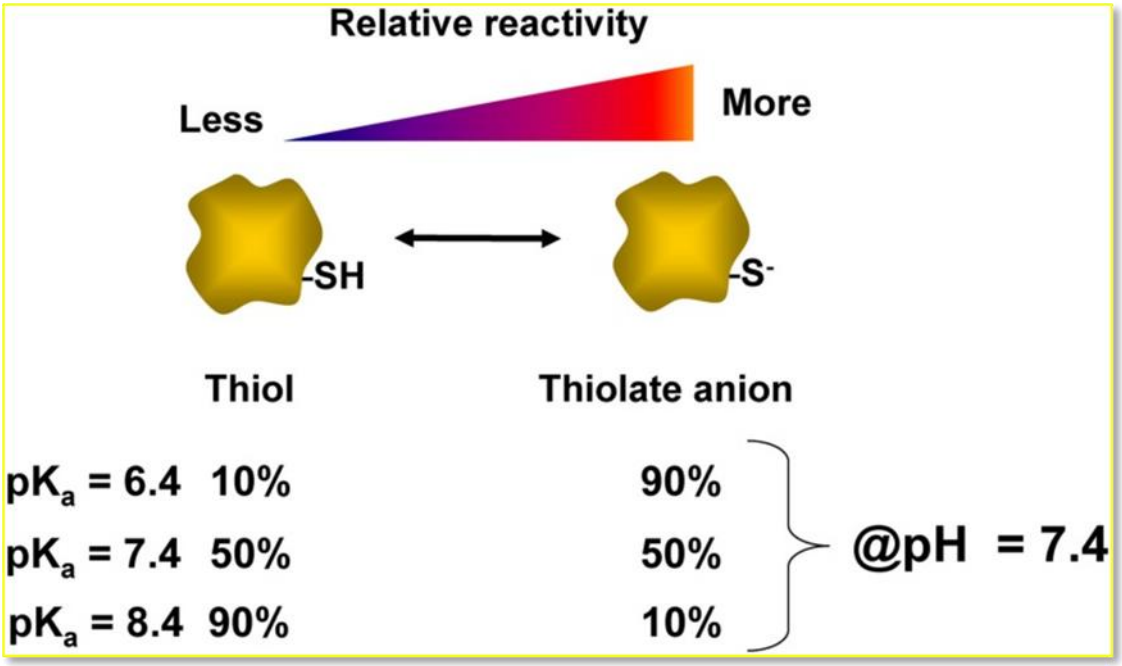


Figure1.8Relationship of thiol pKa and reactivity. The acid dissociation constant (pKa) of a thiol group determines the ratio of thiol:thiolate at a given pH. This value varies for each cysteinyl thiol and is influenced by the local protein environment. At a physiological pH of 7.4, the relative percentages of protonated thiol and deprotonated thiolate anion are listed for cysteine residues which have different pKas of 6.4, 7.4, or 8.4. The thiol with a pKa of 6.4 is expected to be nearly 90% in the thiolate form at pH 7.4, and is therefore more reactive than thiols with higher pKa values[46].

Cysteine and its surrounding environment determines its oxidation state

Cysteine residues form part of enzyme active sites [47].Cysteine SH can also react with nearby SH groups to form a disulfide bridge [47].Cysteine SH groups are also found on the surface of proteins making contact with the external environment. This particular group of solvent exposed or "free" cysteine residues can be modulated to a range of redox-sensitive modifications.

Modification of a solvent exposed SH group depends on its ability to ionize and form a very nucleophilic thiolate anion (S^-) [50]. The pKa of cysteine SH determines the formation of S^-which is influenced strongly by nearby microenvironment. At neutral pH, cysteines is in a protonated and unreactive state(pKa- 8.3) [50]. Alkaline environment increases the probability that a cysteine SH will adopt a deprotonated state .Adjacent positively charged amino acids also can help decrease the pKa of cysteine thiols, making them more reactive. The presence of the partial positive charge of an α-helix dipole or the nitrogen in the peptide backbone can also decrease the pKa of cysteines

[50]. For instance, the catalytic cysteines in glutaredoxin (Grx) isoforms 1 and 2 adopt a very low pKa due to close proximity of a lysine residue and the N-terminus of an α-helix [50].

Similarly, neighbouring negative charges can increase the pKa as in reduced glutathione (GSH) which has a pKa of around 8.8 [50]. As stated SH reactivity also influences whether or not it can adopt various oxidation states; e.g. be reactive enough to detect changes in redox through interactions with H_2O_2. An excellent example of this is peroxiredoxin (Prx) which uses a catalytic cysteine, which has an estimated pKa of 5–6, to metabolize H_2O_2 with great efficiency [50]. Steric hindrance also affects cysteine thiol reactivity since cysteine oxidation reactions are nucleophilic substitution (SN2) reactions [47]. Thus,it is clear that the surrounding environmentdecides whether a thiolate will be reactive enough to undergo modification .Thus for acting as redox switches, Cysteines should be surface exposed and its pKa should be lower than neutral.Many factors like surrounding amino acids,steric hindrance, accessibility of cysteines affect the pKa of cysteine SH.

Oxidative modifications of protein thiols

The thiolate anion of redox-sensitive Cys residues can reversibly oxidize to sulfenic acid or form mixed disulfides or irreversibly oxidize to sulfinic and sulfonic acid [47], which are usually damaging to protein function, although sulfiredoxin has been found to specifically reduce the sulfinic acid moiety in peroxiredoxins[47]

Reversible Oxidation is the key here to protect proteins from irreversible oxidation which may also modulate protein function.

The formation of Disulphides is a kind of reversible oxidation.It can form between two proteins(interprotein)/within a protein(intraprotein),causing changes in protein aggregation and conformation.

Under oxidative conditions, proteins may form interprotein disulphides or intraprotein disulphides causing changes in protein aggregation and conformation. As shown using proteomic techniques, intermolecular disulfide bonds are formed in the cytoplasm upon treatment of cells to oxidative stress [47].

Protein sulfenic acids - main products formed by protein thiols on contact with H_2O_2, are very reactive being frequently an intermediate in sulfinic and/or sulfonic acid generation or reacting with other neighbouring or accessible thiolsto form intra- or intermolecular disulfides, respectively [47]. Despite their reactivity, it is now clear that some sulfenic acids are stable, and can be found in proteins under normal conditions [47]. Protein sulfenate formation playsignificant roles in redox signaling, specially in the redox regulation of transcription factors and enzymes -tyrosine phosphatases, peroxiredoxins, and methionine sulfoxide reductases [47].

Oxidant exposure, in addition to causing irreversible oxidation of Cys residues to sulfonic acids, can lead to excessive disulfide bonding, protein misfolding, and aggregation, as in case of GAPDH aggregation in Alzheimer's disease [47].These covalent aggregates are difficult to reduce even when intracellular redox conditions are restored to normal.

Mixed disulfides, i.e., S-thiolated proteins, form between protein sulfhydryls and low-molecular-mass thiols such as homocysteine, cysteinylglycine, free cysteine, and glutathione [47].

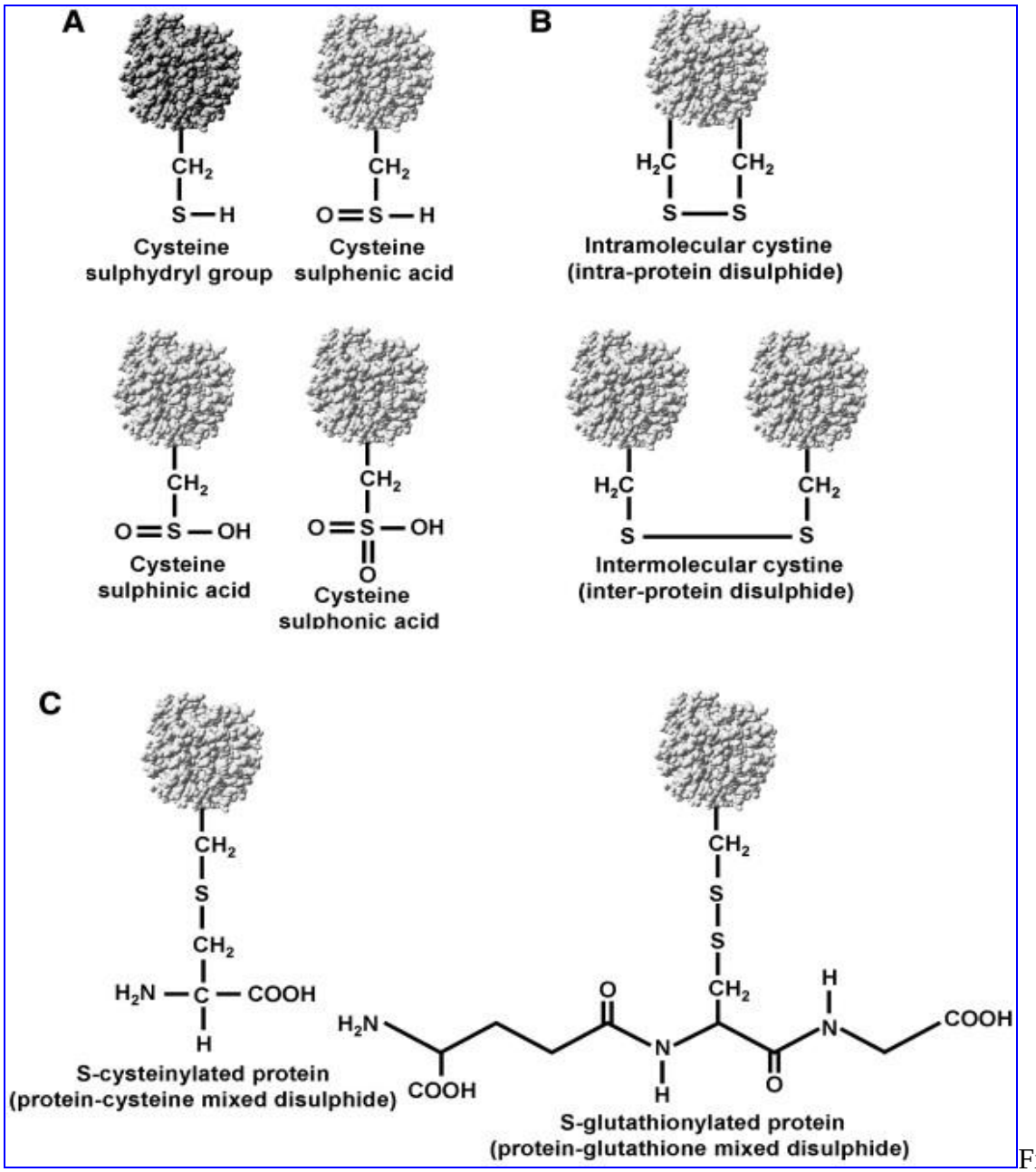


Figure 1.9 Oxidative modifications of protein thiols

(A)The oxidation of a cysteine residue within a protein can result in the formation of a cysteinyl radical (Cys–SU, not shown) or a sulfenic, sulfinic, or sulfonic acid derivative (the latter of which is always irreversible). (B) Alternatively, oxidation can result in a disulfide bridge (cystine). Disulfides can form under oxidative conditions between two adjacent proteins (intermolecular cystine or interprotein disulfide) or between two adjacent sulfhydryl groups within a protein (intramolecular cystine or intraprotein disulfide), causing changes in protein aggregation and conformation. (C) Reaction between protein cysteinyl residues and low-molecular-mass thiols such as glutathione and free cysteine can yield

protein–glutathione or protein–cysteine mixed disulfides, respectively, i.e,S-glutathionylated or S-cysteinylated proteins. Each of these protein thiol modifications has the possibility of eliciting different cellular responses.

(Free Radic Biol Med. 2007 Sep 15;43(6):883-98.[47])

S-Glutathionylation

S-glutathionylation is a kind of reversible post-translational modification of a protein cysteine thiol, with a effective increase in molecular mass of ~ 305.6 (from glutathione, GSH) and a net increase in negative charge (from glutamic acid). It both protects as well as modulates the protein [52]. Low pKa of cysteine (created by the basic three dimensional environment) favours the forward reaction of S glutathionylation.

Mechanism of S-glutathionylation

Protein S-glutathionylation proceeds either spontaneously or enzymatically [52]. The non-enzymatic reaction can proceed as follows:

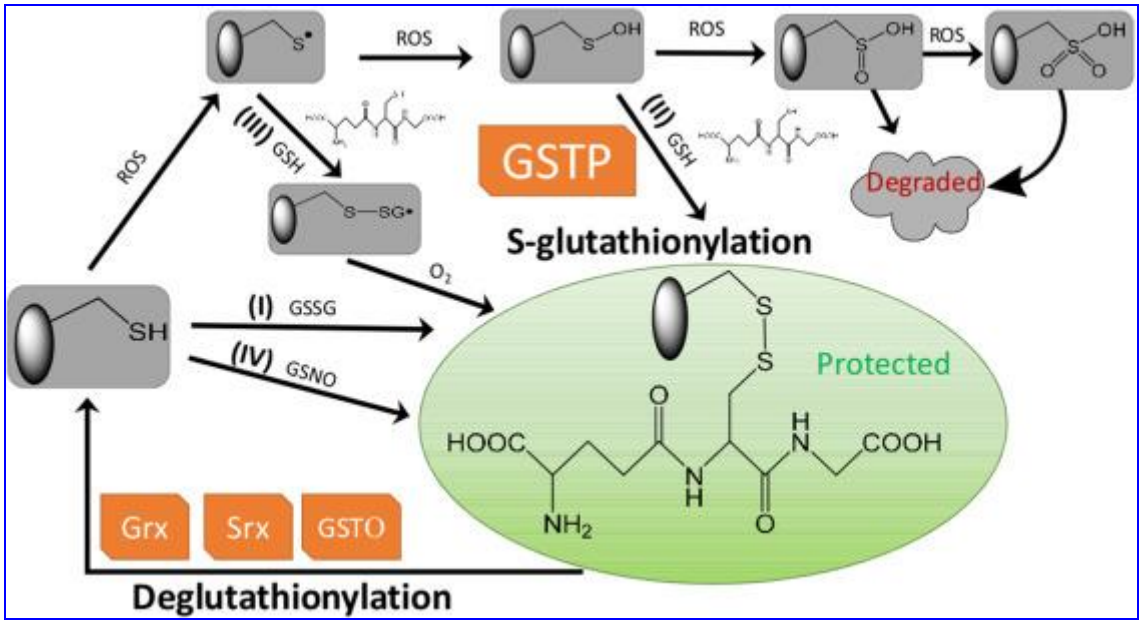
(1) thiol-disulfide exchange between protein thiol (PSH) and glutathione disulfide (GSSG). In this case, the ease of PSSG formation is a product of the equilibrium constant of the reaction (Kmix), expressed as $\frac{[PSSG][GSH]}{[PSH][GSSG]}$. Therefore, the extent of protein s-glutathionylation ([PSSG]/[PSH]) depends on the local GSH:GSSG ratio . For most protein cysteines with Kmix ~ 1, the intracellular GSH:GSSG ratio would have to fall sharply to form PSSG. Such extreme conditions are rare inside the cells, therefore for most proteins, generally PSSG formation through PSH and GSSG exchange is uncommon . However, there are some examples as c-Jun that have a Kmix ~ 13 and therefore 50% of c-Jun may be S-glutathionylated when the GSH:GSSG ratio is ~ 13 [52].

(2) PSH is oxidized by ROS to form sulfenic acid (PSOH) which then rapidly reacts with GSH to form PSSG. Low µM H_2O_2 can rapidly oxidize PSH to -SOH, which is unstable and can further oxidise to sulfinic (SO_2H) and eventually sulfonic acid (SO_3H) which usually irreversibly deactivates the protein. Thus, S-glutathionylation of an SOH group can prevent the target protein from over-oxidation [52]. Under normal physiological conditions, H_2O_2 levels are not high enough to form PSSG through this mechanism usually

(3) PSH is oxidized to a thiyl radical (PS•) which fastly reacts with GSH to form intermediate (PSSG•-) which can then react with O2 to form PSSG. Similarly, GS• can react with PSH to

form PSSG•-. Thiyl radical way of protein S-glutathionylation has been suggested to occur in vivo [52], perhaps involving glutaredoxin 1 and 2 (Grx1 and 2) .

(4) **Nitric oxide (NO) induced S-glutathionylation** Secondary generation of reactive nitrogenspecies (RNS) can promote S-nitrosylation (PSNO) or S-glutathionylation (PSSG) .PSH may also be modified by GSNO to form PSNO and/or PSSG. Previous studies showed that proteins such as papain, creatine phosphokinase or glyceraldehyde-3-phosphate dehydrogenase are susceptible to both S-nitrosylation and S-glutathionylation by GSNO. Furthermore other reports have showed that treatment of cells with PABA/NO (a glutathione S-transferase (GST) activated diazeniumdiolate prodrug) resulted in a dose-dependent increase in intracellular NO levels with high levels of S-glutathionylation of such proteins as, β-lactate dehydrogenase, Rho GDP dissociation inhibitor β, ATP synthase, elongation factor 2, protein disulfide isomerase (PDI), nucleophosmin-1, chaperonin, actin, protein tyrosine phosphatase 1B, and glucosidase II . Interestingly, it has been shown that the S-N bond of GSNO has some polarity, pointing that nucleophilic attack of the protein thiolate anion (PS-) on nitrogen to form PSNO should be favored over sulfur to form PSSG .Taken together, such results proposes that two distinct pools of S-nitrosylated proteins may exist, one that is not affected by GSH and another that is GSH labile and subject to rapid conversion to S-glutathionylated products. However, those properties or conditions that favor the implementation of PSSG versus PSNO are not known at the moment[52]



Fiugre1.10 Protein S-glutathionylation and deglutathionylation cycle. Protein S-glutathionylation proceeds either spontaneously or enzymatically. The non-enzymatic reaction can proceed as follows: (1) thiol-disulfide exchange between protein thiol (PSH) and glutathione disulfide (GSSG); (2) PSH is oxidized by reactive oxygen species (ROS) to a sulfenic acid (PSOH) which then rapidly reacts with GSH to form PSSG, thus prevent the target protein from over-oxidation to sulfinic (SO2H) and eventually sulfonic acid (SO3H) which generally irreversibly deactivates the protein; (3) PSH is oxidized to a thiyl radical (PS•) which rapidly reacts with GSH to form a thiyl radical glutathionyl intermediate (PSSG•-) which can then react with O2 to form PSSG; (4) PSH may be modified by GSNO to form PSNO and/or PSSG. Protein S-glutathionylation can occur spontaneously, but the rates and magnitude are greatly enhanced by enzymes

- [Free Radic Biol Med. 2018 May 20;120:204-216.].

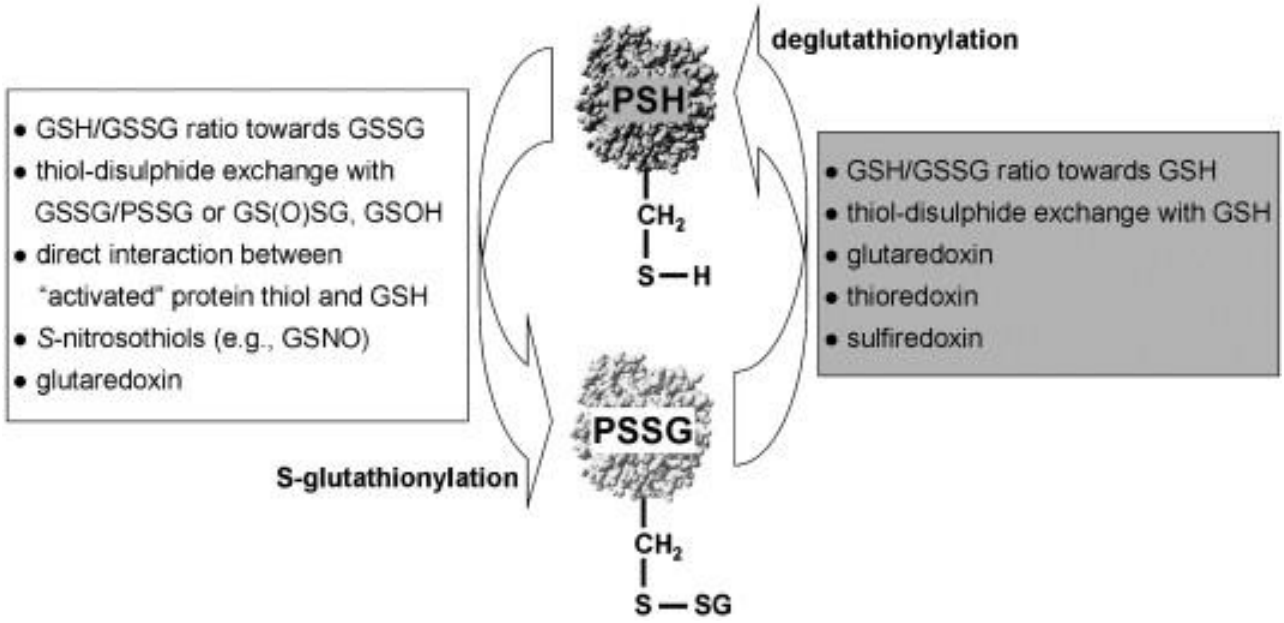


Fig 1.11 Induction and reversal of protein S-glutathionylation. Formation of protein–glutathione mixed disulfides, i.e., S-glutathionylated proteins (PSSG), in biological systems can occur after a decrease in the intracellular/cytoplasmic GSH/GSSG ratio that can induce the partial oxidation of protein sulfhydryls, yielding an "activated" protein thiol (thiyl radical or sulfenic acid). GSH can then interact with this activated thiol to give an S-glutathionylated protein. Alternatively, S-glutathionylation can be the result of thiol/disulfide exchange in the presence of increased cellular levels of GSSG or by other mediators (e.g., GS(O)SG or GSOH). Furthermore, PSSG formation can be promoted by nitric oxide, through various mechanisms including the formation of S-nitrosoglutathione (GSNO) and thiyl radicals. Finally, glutaredoxin can also catalyze the S-glutathionylation of certain proteins in the presence of a GS$^{\bullet}$-generating system via a monothiol mechanism of thiol/disulfide exchange. Reversal of S-glutathionylation (deglutathionylation) can be achieved by changes in the intracellular redox status (increases in the GSH/GSSG ratio), by reduced thiols, or via enzymatic reduction by glutaredoxin (also known as thioltransferase) and sulfiredoxin, which selectively deglutathionylate PSSG by a GSH-dependent or -independent mechanism, respectively, and, in a limited number of proteins, by the thioredoxin system[47].

Enzymes involved in Glutathionylation

The proteins known to catalyze glutathionylation have been shown to be either the glutathione S transferases or the glutaredoxins.

Glutathione S Transferase are enzymes that can conjugate glutathione to numerous substrates. It is known basically for their detoxification system . The GST genes are upregulated in response to oxidative stress and are overexpressed in many tumours. Beside, detoxification ,they also perform glutathionylation of protein for maintenance of cellular redox balance[55].GSTs can be cytosolic ,mitochondrial or microsomal.

Two subgroups of cytosolic GSTs are known in literaturedepending upon their interaction with glutathione: the Y-GST group, which uses a tyrosine residue to activate glutathione, and the S/C-GST, which uses serine or cysteine residues.[54][54]

GSTs have the capacity to lower the pKa of the sulfhydryl group of reduced glutathione (GSH) from 9.0 to about 6.5 when GSH is bound in the active site[55] . GSH forms the thiolate (GS^-) anion at neutral pH when complexed with the GST enzyme[56]. GSTs promote GS^-formation as well as bind hydrophobic electrophilic compounds at nearby site[56] . The GSH-binding and the hydrophobic substrate-binding sites are known as the G- and H-sites.The active cytosolic enzyme exists as a dimer of two subunits[56].

Several GST isozymes prevent the function of kinases involved in the MAPK pathway that controls cell proliferation and death, preventing the kinase from performing its role in facilitating the signaling cascade.[56]

Yeast GSTs : Yeast has 3 reported Glutathione transferases related to the GST omega family.Omega-class GST shows glutathione-dependent thiol transferase and dehydroascorbate reduction activity. This kind of activity has not been observed in any other class of GSTs, but is associated with the glutaredoxins (thioltransferases). Omega class GST have a unique N-terminal extension, and contains a cysteine residue in the active site, which is different from the tyrosine and serine residues found at the active sites of other eukaryotic GSTs[57]These are Gto1p,which is peroxisomal,and Gto2p and Gto3p which are cytoplasmic[57].Two other proteins with a GST domain that has no similarity to mammalian GSTs but are related to the bacterial GSTs are the glutathione transferases,Gtt11p and Gtt2p.Finally, the two glutaredoxins,TheGrx1p and Grx2p have also been reported to have glutathione S-transferase activity.Mutants lacking GRX1 and GRX2 are sensitive to oxidative

stress conditions , whereas mutants lacking GTT1 and GTT2 are unaffected in sensitivity to a range of oxidants and xenobiotics[58].Strains deleted for GTT1 and/or GTT2 are viable but exhibit increased sensitivity to heat shock in stationary phase and limited ability to grow at 39 °C.

The sequence of classic GSTs,GTT1 and GTT2[59] does not allow them to be assigned to any of the classes of GSTs of multicellular eukaryotes[60].

Glutaredoxins

Glutaredoxins , also known as thioltransferases (disulphide reductases), are small proteins of approximately one hundred amino-acid residues which utilise glutathione and NADPH as cofactors.Glutaredoxins are oxidized by substrates, and reduced non-enzymatically by glutathione. Oxidized glutathione is regenerated by glutathione reductase. Together these components compose the glutathione system

Yeast contains a single glutathione reductase which using different translationsal initiation sites generates both cytosolic and mitochondrial counterparts.A deletion in GLR1 drastically increases levels of oxidized glutathione in these two subcellular compartments. GLR1 converts oxidized glutathione to reduced glutathione. Cytosolic Glr1p is the main determinant of the glutathione redox state of the mitochondrial intermembrane space, mitochondrial Glr1p has a role in resistance to hyperoxia[61,62]

Structurally, all GRXs have a 'thioredoxin-fold' structure, which is formed by a four- or five-stranded beta-sheet flanked by three or more alpha-helices on either side of the beta -sheet [60]

Till date, eight Grxs have been found in yeast belonging to two types of glutaredoxins-the dithiol forms with CxxC motif (Grx1, Grx2 and Grx8) and the monothiol forms with CxxS motif (Grx3,Grx4,Grx5,Grx6 and Grx7)[63].Different glutaredoxins have different functions.Grx1p and Grx2p contain a conserved CPYC motif at the active site.Grx1 mutant is hypersensitive to the superoxide anion[61].In contrast both grx1 and grx2 mutants show sensitivity to H_2O_2 with the grx2 mutant showing slightly higher sensitivity .

In *S. cerevisiae*, Grx1 overexpression enhances protection against superoxide anion and hydroperoxides, while Grx2 overexpression allows resistance to hydroperoxides[61] ,which suggests that Both Grxs are partially specialised in their function.

Grx1 exists mostly in cytosol while Grx2 is expressed in two isoforms via alternative translation using different start codons, with the full-length version translocated to the mitochondrial matrix and the truncated version retained in the cytosol[64].

In contrast to these, the monothiol Grxs, Grx3, Grx4, and Grx5, have an active site with the CGFS motif[63].

Nuclear monothiol Grx can substitute for mitochondrial Grx if directed to localise there.Although ,Grx2 could not suppress grx5 mutant phenotype.Basically, Dithiol redoxins are functionally divergent from monothiol ones, but in the latter case, it is the compartmental barrier, that localises their activity[65].

Thus ,the glutaredoxins can have overlapping functions.

In addition to these five well-known yeast Grxs, three new members, Grx6, Grx7, and Grx8 were recently identified[63].Grx6 and Grx7 are monothiol Grxs, with an active-site motif of CSYS in Grx6 and of CPYS in Grx7. These Grxs were proposed to be responsible for regulating the sulfhydryl redox state in the oxidative conditions of early secretory pathway vesicles. Grx3 and Grx4 are nuclear[65] & are required for the regulation of the iron-dependent transcriptional factor Aft1 in the nucleus , iron homeostasis[65] .Grx3 physically interacts with Grx4 in the nucleus [65]. Grx3 has also been shown to localize in the cytoplasm also. Grx3 and Grx4 are highly homologous, and each possesses both an N-terminal Trx-like domain and a C-terminal Grx-like domain [65] and while both the domains perform different functions- each of the two Grx domains plays a role in Ros detoxification and cell viability, while the Trx domain acts independently of their Grx domains in polarization of actin cytoskeleton , which also determines cell resistance against oxidative stress.

Grx5 contains a single Grx domain together with mitochondriol target sequence. The absence of Grx5 causes the inactivation of enzymes containing Fe/S clusters, intracellular iron accumulation, hypersensitivity to external oxidants, high levels of protein oxidation, auxotrophy for a number of amino acids, and low growth rates in rich medium [66]. A number of proteins also become hyperglutathionylated in null grx5 mutants [66]. Grx5 is involved in the synthesis or assembly of Fe–S clusters in the mitochondrial matrix.[66]

Grx6 is localized in both the endoplasmic reticulum and Golgi apparatus, while Grx7 is found mostly in the Golgi apparatus. Grx6, but not Grx7, binds two [2Fe–2S] clusters to form a tetramer in vitro[67] In addition to Grx1 and Grx2, Grx8 is a third putative dithiol Grx, encoded

by GRX8, which does not affect growth rate under oxidative stress conditions when deleted[63].

Deglutathionylation

Glutathionylation can be reversed by deglutathionylation , where GSH is removed from the thiol groups of protein cysteine residues[52].Interestingly although members of the thioredoxin superfamily (that includes thioredoxins, glutaredoxins, sulphiredoxins, GSTs ,GPXs, PDIs) have been demonstrated to display deglutathionylation activity in vitro [52]. However, it is the glutaredoxins that are thought to be the key enzymes principally involved in deglutathionylation in mammals[61].

The case for yeast is different.In, yeast ,thioredoxins are the main deglutathionylation enzymes. Thioredoxins are characterized at the level of their amino acid sequence by the presence of two vicinal cysteines in a CXXC motif.In yeast,Thioredoxin and thioredoxin reductase (TrxR) make up the thioredoxin system , which is essential in the regulation of the intra cellular redox state. Thioredoxin reductase transfer electrons from NADPH to the thioredoxin active site.Reduced thioredoxin itself is oxidised to reduce the target protein and thioredoxin reductase then recyles the disulphides back into its dithiol form.

Yeast contains two genes encoding cytoplasmic thioredoxins (TRX1 and TRX2) which are dispensable during normal growth conditions [43,44]. Yeast mitochondrial thioredoxin system including a thioredoxin (TRX3) and thioredoxin reductase (TRR2), which is thought to function in protection against oxidative stress generated during respiratory metabolism [45]. Deletion of both TRX1 and TRX2 results in a prolonged S phase and shortened G1 interval [44].

Yeast thioredoxins are active as antioxidants and play key roles in protection against oxidative stress induced by various ROS [61]. TRX2 appears to play the predominant role, since mutants lacking TRX2 are hypersensitive to hydroperoxides and mutants containing TRX2 are resistant to these oxidants [61].This requirement for TRX2 is only apparent during stationary phase growth, many evidences indicate that the thioredoxin isoenzymes have redundant activities as antioxidants. For e.g. the trx1 and trx2 mutants show wild- type resistance to hydroperoxide during exponential phase growth;overexpression of either TRX1 or TRX2 leads to a similar increase in resistance to hydroperoxides; and thirdly, both Trx1 and Trx2 are equally able to act as cofactors for the thioredoxin peroxidase Tsa1[68].

The antioxidant activity of thioredoxins is required for both the survival of yeast cells as well as for protection against oxidative stress during stationary phase growth, and correlates with an increase in the expression of both TRX1 and TRX2. At least part of this requirement is dependent on their activity as cofactors with Tsa1 [68].

Mitochondrial thioredoxin system -consisting of TRX3 and thioredoxin reductase (TRR2),which function in protection against oxidative stress generated during respiratory metabolism [68].Since the strains deleted for GRX1, GRX2, TRX1 and TRX2 are lethal , it does not appear that this mitochondrial system can substitute for the cytoplasmic thioredoxin or glutaredoxin systems[61].

A recent study has shown that S. cerevisiae mutants lacking Grx1p and Grx2p don't show high levels of protein glutathionylation , but a trx1 trx2 double deletion mutant shows constitutively high levels of hyper-glutathionylation.The study suggested that thioredoxins are required to maintain protein glutathionylation levels during yeast growth cycle. This is in contrast to other eukaryotic systems ,where glutaredoxins appear to be the predominant deglutathionylase enzymes.Thioredoxins reduce glutathionylated proteins as cells resume exponential phase from stationary phase growth.Therefore, In *S.cerevisiae*,TRX system seems to regulate the glutathionylation state of cellular proteins.

Table given here briefly summarizes the glutathionylation and deglutathionylation enzymes in yeast

Family	protein	Localization	Function
GRXs(Glutaredoxins)	Grx1p,Grx2p	cytoplasm	Protective against oxidative stress
	Grx3p,Grx4p	Nucleus	Actin remodelling, iron homeostasis protection against oxidative stress
	Grx5p	Mitochondria	Protection against oxidativedamage of proteins
	Grx6p,Grx7p	Golgi,ER	Maintains ER redox state
	Grx8p	Cytoplasm	Oxidoredutase activity
TRXs(Thioredoxins)	Trx1p,Trx2p	cytoplasm	Ribonuceotide reductase irreversible protein S-thionylation
	Trx3p	Mitochondria	Regulates mitochondrial redox
GSTs (Glutathione-s-transferase)	Gtt1p,Gtt2p	Cyctoplasm, ER	Arsenic detoxification
	Gto1p	Peroxisomes	GSH-dependent thiol transferases
	Gto2p,Gto3p	Cytoplasm	GSH-dependent thiol transferases
Sulfiredoxins	Srx1p	Cytoplasm	Reductant of sulfinic acid
Peroxiredoxins	Prx1p	cytoplasm	Reduction of alkylhydroperocides

Table1.1: Glutathionylation and Deglutathionylation enzymes in yeast *S.cerevisiae*

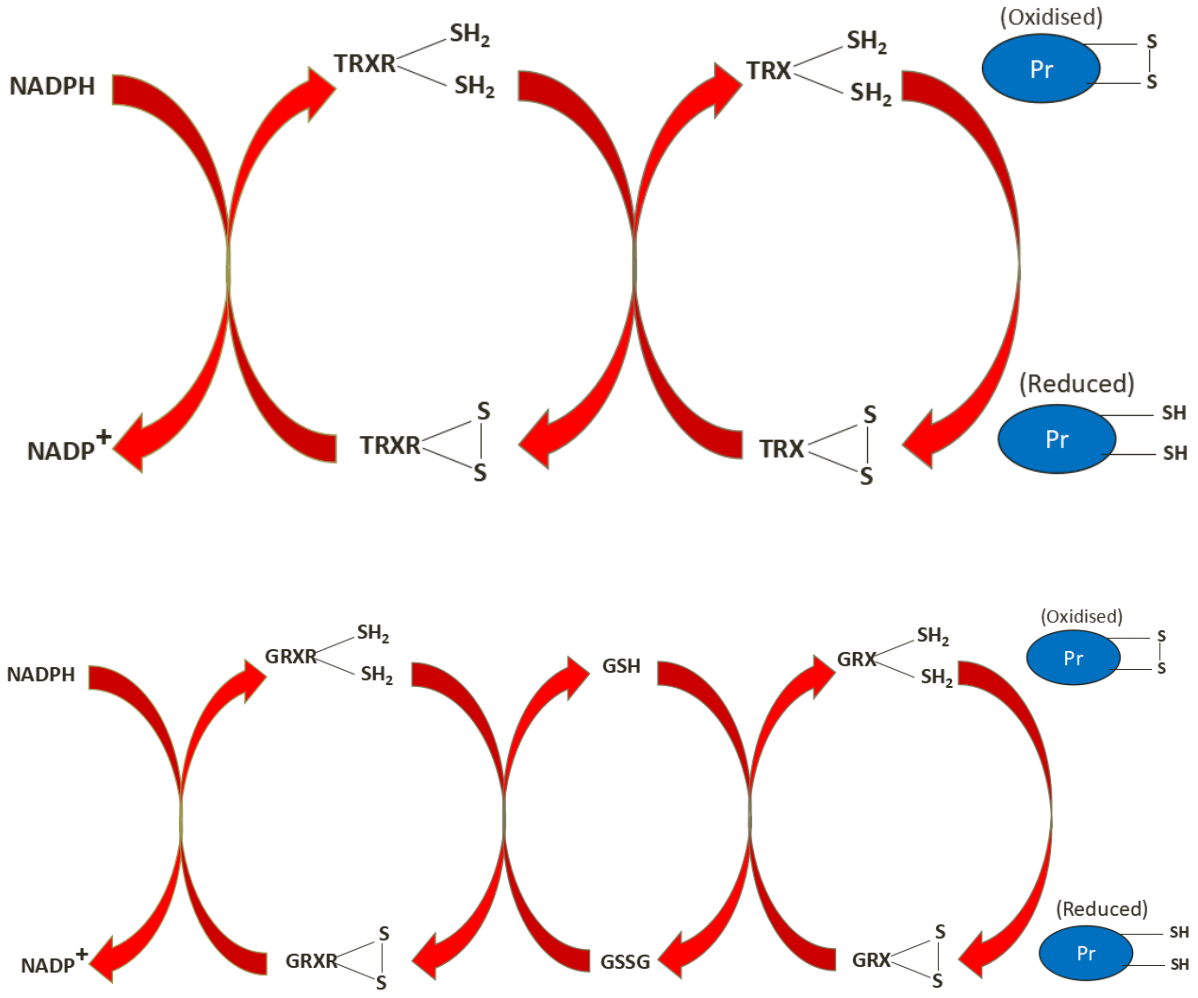


Figure 1.12 Detailed reaction mechanism of thioredoxins and glutaredoxins. The dithiol mechanism (A) oxidoreductase(TRX) is reduced by thioredoxinreductase (TxrR) in the presence of NADPH to recycle reduced thioredoxin which reduces disulphides in the substrate. (B) Reduction of the disulfide bonds in substrate and recycling the reduced glutaredoxin (GRX) by GSH molecules forming glotathione disulfide (GSSG) which is regenerated by glautaredoxinreductase (GrxR) in the presence of NADPH[70]

1.9 Background and Objective

As has been described , it is well known that some glycolytic enzymes get glutathionylated during oxidative stress.A range of glycolytic enzymes -Tdh3p, Eno2p, Adh1p, Tpi1p, Ald6p and Fba1p are identified to be S-glutathionylated.GAPDH, enolase and alcohol dehydrogenase activities were reversibly affected by oxidative stress.While,S thiolation of Fba1,Tpi1 and Ald6 did not affect cellular aldolase,Triose phosphate iosmerase or aldehyde dehydrogenase activities were unaffected.Specific glutathionylation of yeast mitochondrial proteins under stress and basal conditions have shown phosphoglycerate kinase and enolase are glutathionylated.

There are several unanswered questions related to the glutathionylation of glycolytic enzymes.

First, Do these proteins show glutathionylation under normal conditions also? Do these proteins function as a source of storage of glutathione.

Second, do the glutathionylation only occur during oxidative stress and do the glutathionylation levels of these proteins change in response to oxidatvie stress?

Third, Is this a protective mechanism or regulatory or both ?

Fourth, Which are the enzymes involved in the glutathionylation /deglutathionylation of these proteins?

Lastly, are there overlaps in controlling mechanisms? For example,does a single glutaredoxin carry out glutathionylation of all the glycolytic enzymes or there are different enzymes devoted to control a single pathway.

With these questions, I formulated the objectives of my thesis as follows:

1. To clone and express the glycolytic enzymes which are glutathionylated in yeast.

2. To evaluate the glutathionylation of these enzymes in stress vs non-stress conditions

3. To determine the reversibility of the glutathionylation reaction

4. To evaluate the enzymes involved in the glutathionylation or deglutathionylation of glycolytic enzmyes

5. To determine the mode of regulation by these enzmyes and their effect on activity

Chapter 2: Materials and methods

SECTION A: Materials

2.1 Chemicals and Reagents

All chemicals used were obtained from commercial sources and were of analytical grade.Media components, fine chemicals and reagents were purchased from Sigma Aldrich (St.Louis, USA), HiMedia (Mumbai, India), Merck. India Ltd (Mumbai, India), USBCorporation (Ohio, USA) or Difco, USA. Oligonucleotides (primers) were designed usingSnapGene (version 2.4.3) software and were purchased from Integrated DNATechnologies (IDT). Enzymes (Restriction enzymes, T4 DNA ligase, Calf IntestinalPhosphatase (CIP), Vent DNA polymerase, Phusion High-fidelity

DNA polymerase and other modifying enzymes), their buffers, dNTPs, DNA and proteinmolecular weight markers were purchased from New England Biolabs Inc, (Beverly, MA,USA) or Thermo Scientific. Gel-extraction kits and plasmid miniprep columns wereobtained from QIAGEN or Fermantas.PVDF membrane and ECL Plus Western Blotting Detection Reagents were purchased from Amersham Biosciences GE health care(U.K.). His-Tag mouse monoclonal antibody and goat anti-Mouse HRP-linked antibody were bought from Cell Signaling (Danvers, MA, USA).

2.2 Strains and Plasmids

Escherichia coli DH5α was used as the cloning host. The genotype for the *E. coli* and *Saccharomyces cerevisiae* strains used in the study are given in Table 2.1.The E.coli strain DH5α was used as a cloning host and grown at 30°C.The list of various plasmids used in this study is given in Table 2.2.

Table 2.1: List of bacterial and yeast strains used in the study

Strain	**Genotype**	**Source**
Escherichia coli strains		
ABE 460 (DH5α)	F - *gyr A96(Nal) recA1 relA1 endA1 thi-1 hsdR17*(r_{k-} m_{k+}) *gln V44* *deoR Δ(lacZYA-argF) U169 [ϕ80dΔ(lacZ) M15]*	Lab strain
Saccharomyces cerevisiae strains		
ABC 733 Wild Type(WT) (BY4741)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Lab stock
ABC 3093 (BY4741 grx1 Δ)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 grx1::KanMX4*	Euroscarf Y06681
ABC 3092 (BY4741 grx2 Δ)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* *grx2::KanMX4*	Euroscarf Y04347
ABC 3090 (BY4741 trx1 Δ)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YLR043c::KanMX4*	Euroscarf Y02654
ABC 3091 (BY4741 trx2 Δ)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 trx2::kanMX4*	Euroscarf Y04839
ABC 4835 (BY4741gtt1 Δ)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* *gtt1::kanMX4*	Euroscarf Y05973
ABC 4836 (BY4741gtt2 Δ)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* *gtt2::kanMX4*	Euroscarf Y01548
ABC1626 (BY4741gto1 Δ)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* *gto1::kanMX4*	Euroscarf Y04784
ABC2358 (BY4741gto2 Δ)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* *gto2::kanMX4*	Euroscarf Y05992
ABC2359 (BY4741gto3 Δ)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf Y00836

	gto3::kanMX4	
ABC 1091 (BY4742 grx3 Δ)	*MATalpha ura3Δ his3Δ leuΔ lys3Δ grx3::kanMX2*	Euroscarf deletion in BY4742 background
ABC1109 (BY4742 grx4 Δ)	*MATalpha ura3Δ his3Δ leuΔ lys2Δ grx4::kanMX2*	Euroscarf deletion in BY4742 background
ABC 1108 (BY4742 grx5 Δ)	*MATalpha ura3Δ his3Δ leuΔ0 lys2Δ grx5::kanMX2*	Euroscarf deletion in BY4742 background

Table 2.2: List of plasmids used in the study

Plasmid name	**Clone no.**	**Description**
Vectors		
p416TEF	ABE 443	The CEN-vector bearing URA3 marker and TEF Promoter-MCS terminator for yeast expression and Amp_r marker for selection in *E.coli*. (Mumberg, et al., 1995)
PGK1-p416TEF	ABE5887	Gene cloned between Bam1 and Xho1 sites
ENO2-p416TEF	ABE5886	Gene cloned between Bam1 and Xho1 sites
TPI1-p416TEF	ABE5888	Gene cloned between EcoR1 and Xho1 sites
FBA1-p416TEF	ABE5885	Gene cloned between Bam1 and Xho1 sites
CDC19-p416TEF	ABE5860	Gene cloned between Bam1 and Xho1 sites
TDH3-p416TEF	ABE5859	Gene cloned between EcoR1 and Xho1 sites

2.3. Oligonucleotides

The list of various oligonucleotide primers used in this study is given in Table 2.3.

Table 2.3: List of Oligonucleotides (and their sequences) used in this study

Oligonucleotide name	Sequence (5' to 3')
PGK1 Forward Primer	CGCAGCGGATCCATGTCTTT ATCTTCAAAGTTGTC
PGK1 Reverse Primer	AGCCGGCTCGAGTTAGTGGT GATGGTGATGATGTTTCTTTT CGGATAAGAAAGCAACACC
FBA1 Forward Primer	ATCGCTCTAGACTTGTGCGT TATTTGTTTTGATTACTAGGC
FBA1 Reverse Primer	ATCGCCATATGATGGGGGAA AAAAGGACAATAATCAAAG CGC
TDH3 Forward Primer	GACCAGGAATTCATGGTTAG AGTTGCTATTAA
TDH3 Reverse Primer.	GATACCTCGAGTTAGTGGTG ATGGTGATGATGAGCCTTGG CAACGTGTTC
CDC19 Forward Primer	CACGGCGGATCCATGTCTAG ATTAGAAAGATTGACC
CDC 19 Reverse Primer	ACGTCGCTCGAGTTAGTGGT GATGGTGATGATGAACGGTA GAGACTTGCAAAGTGTTGG
TPI Forward Primer	GGCCACGAATTCATGGCTAG AACTTTCTTTGTCG
TPI Reverse Primer	GGCACGCTCGAGTTAGTGGT GATGGTGATGATGGTTTCTA GAGTTGATGATATCAAC
ENO2 Forward Primer	GAACGCGGATCCATGGCTGT CTCTAAAGTTTACG
ENO2 Reverse Primer	CAGCCGCTCGAGTTAGTGGT GATGGTGATGATGCAACTTG TCACCGTGGTGGAAG
TEF For. (sequencing)	TTGATATTTAAGTTAATAAA CGG

TEF Rev. (sequencing)	TTCAGGTTGTCTAACTCCTTC

2.4 Media

All the media, buffers and stock solutions were prepared using Millipore elix 5 deionized water unless otherwise mentioned. They were sterilized, as recommended, either by autoclaving at 15 lb/inch^2 (psi) pressure at 121ºC for 15 minutes, or by using membrane filters (Advanced Microdevices Pvt. Ltd., India) of pore size 0.2-0.45 µm (for heat labile compounds). Agar was added, if required, at a final concentration of 2.2%.Ampicillin was added at a final concentration of 100µg/mL.

2.4.1. LB Medium (Luria–Bertani) (per 1000 mL)	Yeast extract 5 g/L Tryptone 10 g/L NaCl 10 g/L [pH was adjusted to 7.0 with 1N NaOH]
2.4.2. YPD Medium (Yeast extract Peptone Dextrose) (per 1000 mL)	Yeast extract 10 g/L Peptone 20 g/L Dextrose 20 g/L
2.4.3 SD Medium (Synthetic Defined) (per 1000 mL)	YNB(Yeast Nitrogen Base) 1.7 g/L (without amino acids and ammonium sulphate) $(NH_4)_2 SO_4$ 5 g/L Dextrose 20 g/L Amino acids (as required) 80 mg/L [pH was adjusted to 6.0–6.5]

2.5 BUFFERS AND STOCK SOLUTIONS

2.5.1 Ampicillin (100 mg/mL) The required amount of ampicillin (sodium salt) was dissolved in the required volume of
deionized water, and was filter-sterilized using 0.2µm membrane filter.

2.5.3 50% Glycerol (used for preparing –80°C stocks of E.coli)

50 mL glycerol is dissolved in 50 mL of deionized water and mixed properly. The solution was autoclaved and stored at room temperature.

2.5.3 25% Glycerol (used for preparing –80°C stocks of yeast)

2.5.5 Alkaline Lysis Buffers (Plasmid DNA preparation fromE.coli)

a) Solution-I (Resuspension Solution)	50 mM Glucose 25 mM Tris-HCl (pH 8.0) 10 mM EDTA (pH 8.0) Autoclaved and stored at 4ºC.
b) Solution-II (Lysis Solution)	0.2N NaOH (freshly diluted from a 10N stock)
(freshly prepared)	1% SDS (freshly diluted from a 10% stock) Stored at room temperature.
c) Solution-III (Neutralization Solution)	5M Potassium acetate 60 mL Glacial acetic acid 11.5 mL Deionized water 28.5 mL The resulting solution is 3M with respect to potassium and 5M with respect to acetate. It was stored at 4ºC.
d) TE Buffer (Tris-EDTA) (pH 8.0)	10 mM Tris-HCl (pH 8.0). 1 mM EDTA (pH 8.0).
e) TE-RNAse (stock prepared at 10 mg/mL)	Working stock 20 µg/mL in TE Buffer, pH 8.0.

2.5.6 Agarose Gel Electrophoresis Reagents

a) 1× TAE (Tris-acetate-EDTA)	40 mM Tris-acetate. 1mM EDTA (pH 8.0).

Buffer (per 1000 mL) (prepared from 50× TAE stock)	Autoclaved and stored at room temperature.
b) Orange-G dye (Gel loading dye, 6X)	0.25% orange-G 30% glycerol
c) 0.7-1% Agarose gel in 1× TAE	
d) Ethidium Bromide (10 mg/mL) Stock	Final working concentration used at 0.5µg/mL.

2.5.7 Solutions for preparation of chemical competent *E.coli* cells

a) SOB	Bactotryptone 20 g Bacto yeast extract 5 g NaCl 0.5 g Above mentioned components were dissolved in 950 mL of water. 10 mL of 250 mM KCl was added and pH was adjusted to 7 with 5N NaOH, volume was made up to 995 mL and autoclaved. Just before use, 5 mL of filter sterilized 2 M $MgCl_2$ was added.
b) SOC	SOB + 20 mM Glucose
c) 10% glycerol	

2.5.8 Yeast Transformation Solutions (*S. cerevisiae*) [75]

a) 0.1M Lithium acetate in TE (pH 7.5)

b) 50% PEG-3350 in 0.1 M Lithium acetate in TE (pH 7.5).

2.5.9 Breaking buffer for genomic DNA isolation from yeast

10 mM Tris-HCl (pH 8.0)

1 mM EDTA (pH 8.0)

100 mM NaCl

1% (w/v) SDS

2% (v/v) Triton X-100

2.5.10 SDS-PAGE Solutions and Reagents [75]

a) 30% Acrylamide Mix	29.2% (w/v) acrylamide 0.8% (w/v) N, N'-methylenebisacrylamide Filtered before use.
b) Resolving (Lower) Gel Tris Buffer	18.18 g Tris
(Stock) (4×) 1.5 M Tris-HCl (pH 8.8)	pH adjusted to 8.8 with 6N HCl and volume made up to 100 mL. (0.4% SDS can be added to the buffer itself).
c) Stacking (Upper) Gel Tris Buffer (Stock) 0.5 M Tris-HCl (pH 6.8)	6.06 g Tris pH adjusted to 6.8 with HCl and volume made up to 100 ml. (0.4% SDS can be added to the buffer itself).
d) 0.4% SDS	
e) TEMED	
f) APS (Ammonium persulphate)	10%
g) Tris-Glycine Gel Running Buffer (Laemmli Buffer) (pH 8.3)	25 mM Tris base 250 mM Glycine (electrophoresis grade) 0.1% SDS
h) 2× SDS Gel Loading Buffer (pH 6.8)	100 mM Tris-HCl (pH 6.8) 4% (w/v) SDS 20% (v/v) Glycerol

	200 mM β-mercaptoethanol (add immediately before use) 0.2% (w/v) Bromophenol blue
i) Gel Staining Solution	40% (v/v) Methanol 10% (v/v) Glacial Acetic acid 50% (v/v) Distilled water 0.1% Coomassie Brilliant Blue (R250)
j) Gel Destaining Solution	40% (v/v) Methanol 10% (v/v) Glacial Acetic acid 50% (v/v) Distilled water

Composition of SDS-PAGE Resolving (Lower) Gel (10%) (For 5 mL)

a) Distilled water	1.9 mL
b) Resolving (Lower) Gel Tris Buffer (Stock) (4×) 1.5 M Tris-HCl (pH 8.8)	1.3 mL
c) 30% Acrylamide Mix	1.7 mL
d) 10% SDS	0.05 mL
e) 10% APS (Ammonium persulphate)	0.05 mL
f) TEMED (N, N, N', N'-Tetramethylethylenediamine)	0.01 mL

Composition of SDS-PAGE Stacking (Upper) Gel (5%) (For 3 mL)

a) Distilled water	2.1 mL
b) Stacking (Upper) Gel Tris Buffer (Stock) 1M Tris-HCl (pH 6.8)	0.38 mL

c) 30% Acrylamide Mix	0.5 mL
d) 10% SDS	0.03mL
e) 10% APS (Ammonium persulphate)	0.03mL
f) TEMED (N, N, N', N'-Tetramethylethylenediamine)	0.003 mL

2.5.11 Immunoblotting (Western Blotting) Reagents [75]

a) Transfer Buffer (pH 9.2)	48 mM Tris base 39 mM Glycine 0.037% SDS 20% Methanol
b) Tris-Buffered Saline (TBS)	150 mM NaCl 20 mM Tris-HCl (pH 7.5) (Made as 10× stock and kept at 4°C)
c) Washing Buffer TBS-Tween 20 (TBST) Buffer	0.1% Tween 20 in TBS (pH 7.5)
d) Blocking Agent	5% Skim milk in TBST.

SECTION B: METHODS

2.6 Identification of glycolytic enzymes known to be glutathionylated in yeast

Literature search was done to find glycolytic enzymes which get glutathionylated .The properties of these enzymes were enlisted later.

2.7 Growth and maintenance of bacteria and yeast strains

The *Escherichia coli* DH5 alpha strains were routinely grown in LB medium at 37ºC at 220 rpm. *E.coli* transformants were selected and maintained on LB medium supplemented withampicillin.

The *Saccharomyces cerevisiae* strains were regularly maintained on YPD medium andgrown

at 30ºC at 220 rpm. The yeast transformants were selected and maintained on SD mediumwith amino acid supplements as per requirements.

2.8 Recombinant DNA methodology (restriction digestion, ligation, transformationof *E. coli.* PCR amplification, etc)

All the molecular techniques used in the study for manipulation of DNA, protein, bacteriaand yeast were according to standard protocols or as per manufacturer's protocol,unless specifically mentioned. DNA sequencing was done usingAgriGenome sequencing services.

2.9Transformation of yeast

The transformation of *S. cerevisiae* strains was carried out by lithium acetate method [8]. *S. cerevisiae* cultures were grown in YPD at 30°C with shaking at 220 rpm for 12-16 hrs andthen reinoculated in fresh YPD to an initial OD600 of 0.1, cells were allowed to grow at30°C for 4-6 hrs with shaking. Cells were harvested at 6000 rpm for 5 min, then werewashed with sterile water and suspended in 0.1M 1ml lithium acetatesolution (prepared in TE, pH 7.5) and were left for incubation for 30 minutes Then the cells were spun down, suspended in 0.1 M 50µL lithium acetate solution to a cell density of1□109 cells/mL. Approximately 100 µg (10µL of 10 mg/mL
stock solution) of heat denatured, salmon sperm carrier DNA, 0.3µg- 0.7µg of
plasmid/DNA fragment and 0.3mL of 50% PEG 3350 (prepared in 0.1M lithium acetate,pH 7.5) were added to each aliquot and whole cell suspension was subjected to heat shock for 5 min after keeping the mixture for 45 min at room temerature.The cell suspensions were allowed to cool to room temperature. The
cells were pelleted down at 5000 rpm for 2 min. The cell pellet was washed with 1 mL waterand resuspended in sterilewater(100 µL) and appropriate volume of cell suspension was plated on selection plates.

2.10 Isolation of genomic DNA from yeast

Genomic DNA from *S. cerevisiae* strains was isolated as described using the glassbead lysis method and the Breaking buffer[76]

2.11 Western blot analysis of C-terminal 6X His tagged proteins

Equal amount of protein samples were loaded on a 10% SDS–polyacrylamide gel(depending upon the protein sizes used), alongwith pre-stained ladder. The gel was run at 100 V.The gel

was electro-blottedonto PVDF (Amersham Bioscience) at 90V for 1 hrsusing Tris-glycine methanol transfer buffer. Immediately after the transfer, the membrane wasincubated at for 2 hrs in blocking buffer (5% skim milk in TBST buffer). After blockingthe membrane it was probed with mouse anti-his primary antibody at a dilution of 1:3000in blocking buffer for 2 hr at room temperature or 4℃ overnight. After 3×5 minutes washing of the blotwith TBST, the membrane blot was incubated for2 -4 hr in anti-mouse (horseradishperoxidase-conjugated) antibody at a dilution of 1: 8000 in blocking buffer. After 3×5minutes washing of the blot with TBST, the blot was sprayed with chemi-luminescentHRP substrate. The signal was detected in LAS Imaging System.

2.12 Method for analysing glutathionylation/deglutathionylation of proteins in vivo

Secondary cultures(starting volume-300 ml) of respective deletion strains were put at 0.1 OD. Cells harvested at 0.8-0.9 OD were pelleted down ,washed with water ,divided into 3 samples -Control(C) ,Test(T) ,Degluthionlyation in water/media(DM/DW) after 2 mM H_2O_2 stress. 2mM H_2O_2 stress for 20 min in dark were given to both T & DM/DW. After that all the three samples were pelleted, washed with water ,C & T were freezed at -80 °C .DM/DW was left to deglutathionylate in water or media for 2 hours ,after that it was also pelleted and freezed at -80 °C. Next day samples were lysed in Protein Extraction Buffer (pH 8.0) 500 µl using beads by agitating 20 cycles on/off (30 sec on bead beater ,30 sec on ice).The sampes were finally centrifuged at 4 °C ,10,000 rpm for 20 min and the supernatant was collected in new MCT and samples were freezed at -80 °C for storage.Supernatant was incubated with 500 µl Ni-NTA agarose(Qiagen) at 4°C for 3-4 hours.Samples were cooled for 10 min on the ice before centrifugation at 1000 rpm for 1 min.Pellets were resuspended in 1 ml ice-cold wash buffer (50 mM Tris,pH 8.0,10 %(v/v) glycerol, 30 mM imidazole pH 6.5,1.5 mM PMSF,PIC) and the centrifugation was repeated.This was repeated twice.The slurry was resuspended in 200 µl of elution buffer (50mM Tris,pH 8.0,10 %(v/v) glycerol, 300 mM imidazole pH 6.5,1.5 mM PMSF,PIC) and incubated at 4 °C with gentle shaking for 5 min.Finally slurry was centrifuged at 1000 rpm for 5 min,and the eluted protein supernatant was collected.This was repeated twice.

Equal amount of protein samples denatured in (100 mM Tris-HCl, pH-8, 4% (w/v) SDS, 20% glycerol(v/v),0.004% (w/v) bromophenol blue at 80 °C for 10 min were resolved by non-reducing SDS/PAGE (10% acrylamide gel),electroblotted onto PVDF membrane and was probed with mouse monoclonal Anti-His and Anti-GSH primary antibody at the dilution of 1:3000.The proteins were finally probed with goat anti-mouse HRP conjugated secondary IgG and visualized using chemiluminescence detection reagent.The levels were compared .

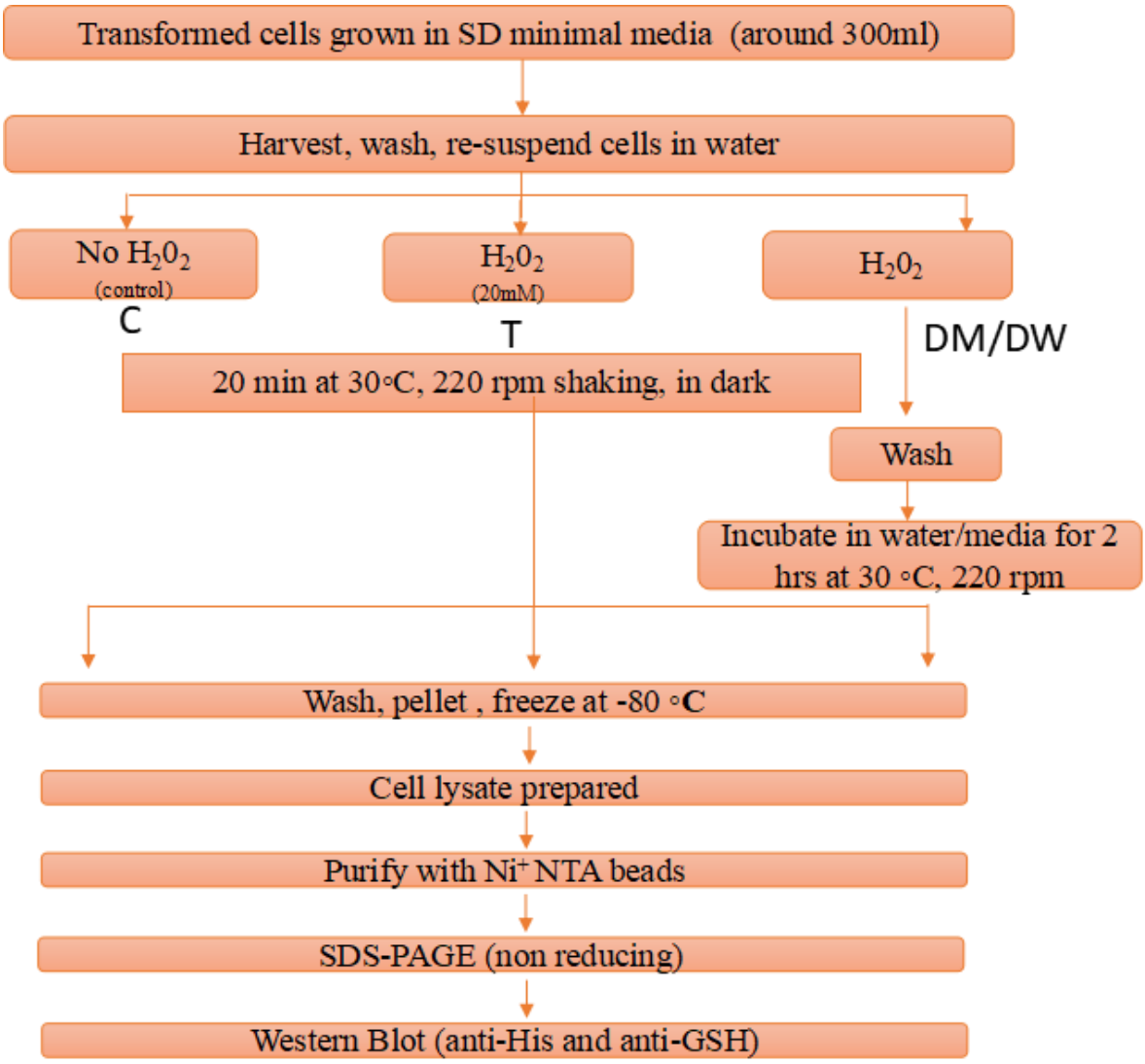


Figure2.11 Flowchart forevaluation of glutathionylation of Proteins

2.13 HU buffer lysis protocol

To 2 O.D. cells of rapidly dividing cells(0.8-1 O.D.), of yeast (pelleted down), 900 µl **of 2N NaOH were added and kept in ice for 10 min.**

After that 200 µl 55% TCA(Trichloroacetic acid in water) was added to each sample in dark, mixed and kept in ice for 10 min.Then the samples were spinned at 4℃ and all the supernatant was carefully removed.The pellet was dissolved in 40 µl HU buffer ,heated at 65 ℃,1400 rpm and 10-20 µl was directly used for non-reducing denaturing SDS-PAGE.This method was good for checking expression of taggged protein but not for checking glutathionylation.

2.14 Sequence Analysis

All the protein sequences were retrieved from Uniprot.The sequences were analysed for the number of cysteines present in it.

The snapgene software was used for restriction analysis, oligo analyis and for introducing point mutations in the coding sequence of the genes.

2.15 Structure Visualisation

All the protein structures were visualised using Pymol by PDB file accessed from RCBS.The proximity of cysteines to surface as well as to active site was looked upon.

Chapter 3: Results and discussion

3.1 Identification of yeast glycolytic genes known to be glutathionylated

By literature mining, we first prepared a list of all those glycolytic enzymes showing glutathionylation in stressed conditions(Refer Table 3.1).The irreversible oxidation of cysteine residues present in a protein can be prevented by protein S-thiolation ,a process by which -SH groups form mixed disulphides with low molecular weight thiols such as glutathione[27,66].During oxidative stress , Earlier reports have shown that several glycolytic enzymes gets glutathionylationed in response to oxidative stress[27]. Glyceraldehyde -3-phosphate dehydrogenase(Tdh3),Phosphopyruvte dehydratase(Eno2),Triose phosphate isomerase(Tpi1),Aldehyde dehydrogenase(Ald6),Fructose -bisphosphate aldolase(Fba1) were shown to be glutathionylated [66].

The first question we asked which are the glycolytic enzymes,that are getting glutathinoylated in yeast?

We also examined the number of cysteines in each of them as this may point towards towards the location and incidences of glutathionylated sites in the enzyme.

Pyruvate kinase (Cdc19) & Fructose bisphosphate aldolase(Fba1) has the highest number of cysteines,while Tdh3, Tpi1 has 2 each and pgk1 and eno2 has a single cysteine .

Gene	Full Name	Protein Size (Amino Acids)	Cysteines	Subunit Structure
CDC19/PYC1	Pyruvate KInase	500	7	Homo tetramer
FBA1	Fructose bisphosphate aldolase	359	5	Homo-Dimer
TDH3	Glyceraldehyde-3-phosphate dehydrogenase	332	2	Homo-Tetramer
PGK1	3-Phosphoglycerate kinase	416	1	Monomer
TPI1	Triose phosphate Isomerase	248	2	Homo-Dimer
ENO2	Phosphopyruvate hydratase	437	1	Homo dimer

Table3.1 Yeast glycolytic enzymes shown to be glutathionylated : Cysteine & subunit structure.Pyruvate kinase contained the highest number of cysteines -7 ,Fba1p has 5,Tdh3p and Tpip has 2 each, while Pgk1p and Eno2p has 1 each

Gene	Gene Size (bp)	promoter	Cloning Site	6X His Tag	Sequence verifed
CDC19	1545	TEF	BamH1 Xho1	C-terminus	✓
FBA1	1119	TEF	BamH1 Xho1	C-terminus	✓
TDH3	1035	TEF	EcoR1 Xho1	C-terminus	✓
PGK1	1293	TEF	BamH1 Xho1	C-terminus	✓
TPI1	789	TEF	EcoR1 Xho1	C-terminus	✓
ENO2	1356	TEF	BamH1 Xho1	C-terminus	✓

Table3.2: Details of cloned genes and Sequencing.The clones were made in p416TEF vector.All the sequences were confirmed correct by restriction digestion and sequencing.

3.2 Cloning of Six glycolytic genes of yeast

The genes encoding the glycolytic enzymes known to be glutathionylated in yeast were cloned in a yeast expression vector p416 under TEF promoter.A 6x His -tag was attached at the C-terminus of all the genes in order to later purify the protein.The coding sequences for the genes were obtained from SGD(Saccharomyces Genome Database).The genes were amplified from genomic DNA and cloned into yeast expression vector All the clonings were confirmed by restriction digestion and sequencing using TEF forward and reverse primers(Fig.3.1)

Glycerol stocks of clones were prepared and stored at -80ºC for further use.

3.3 Recombinant expressionof glycolytic enzmyes

The p416TEF plasmids carrying the genes encoding the different glycolytic enzymes were transformed in WT *Saccharomyces cerevisiae* (BY4741genotype- MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0).

2 -4 O.D. cells of each strain were lysed using modified NaOH lysis/TCA precipitation method and 10-20 µl was directly used for non-reducing denaturing SDS-PAGE .

Out of Tdh3, Eno2, Fba1, Cdc19, Pgk1and Tpi1, only three - Pgk1, Tpi and Fba1could be detected in crude lysate(Fig3.3)Various reasons could be possible if the protein not detected -His tag may not be accessible,protein may be getting degraded.

We proceeded with three proteins that we have confirmed for expression .We purified these three his tagged proteins using Ni-NTA beads.

Figure3.2 Restriction Digestion to verify the clones.p416TEF size is around 5512 base pairs.All the restriction digestion confirmed to the correct gene size of respective gene cloned.

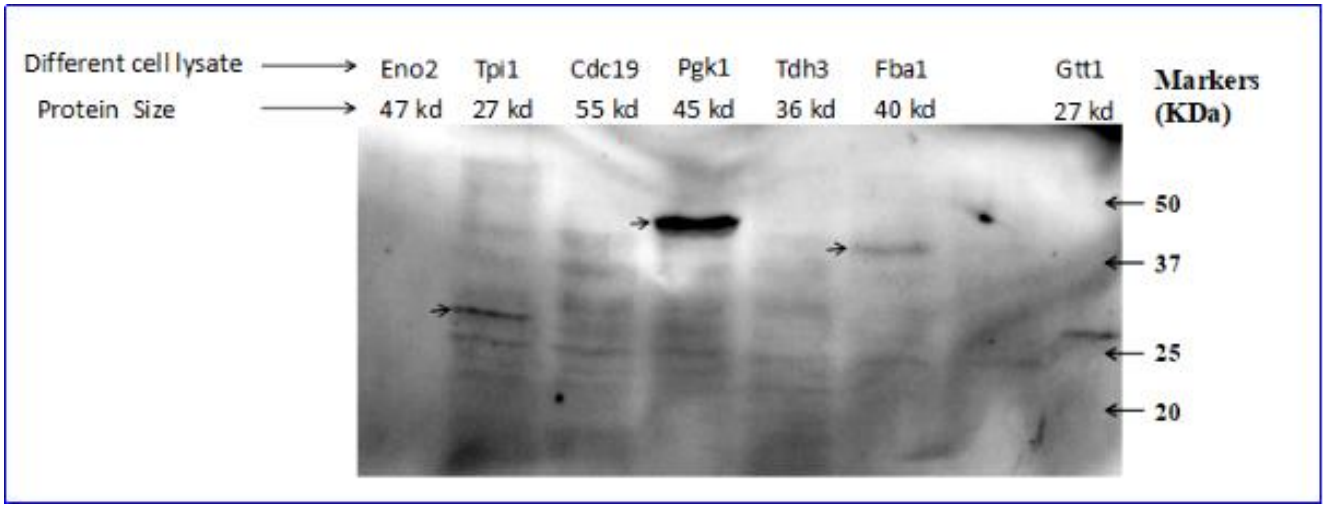


Figure3.2 Non-reducing Denaturing Western Blot of cell lysates probed with Anti-His antibody.Cell lysates were denatured under non-reducing conditions (without DTT) and run on 12% SDS gel .After that it was trasferred to PVDF membrane and probed with mouse monoclonal primary Anti-his antibody and then with goat anti-mouse HRP conjugated IgG secondary antibody .

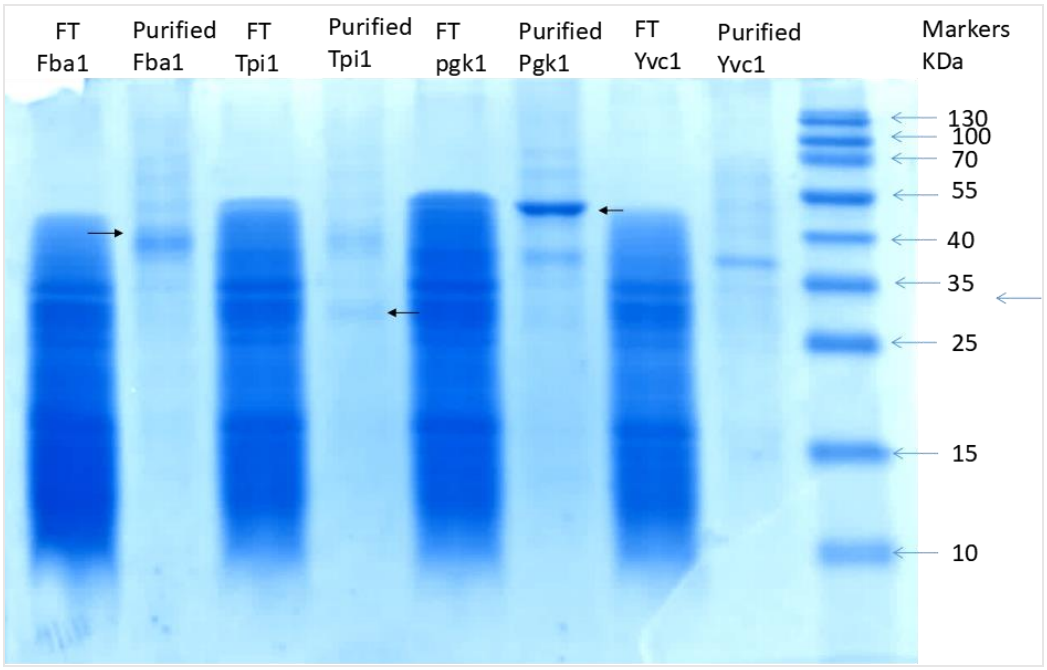


Figure3.3 Flowthrough(FT) & Elutions of Ni-NTA purified proteins .SDS gel was stained with coomasie blue stain overnight and destained in destaining solution

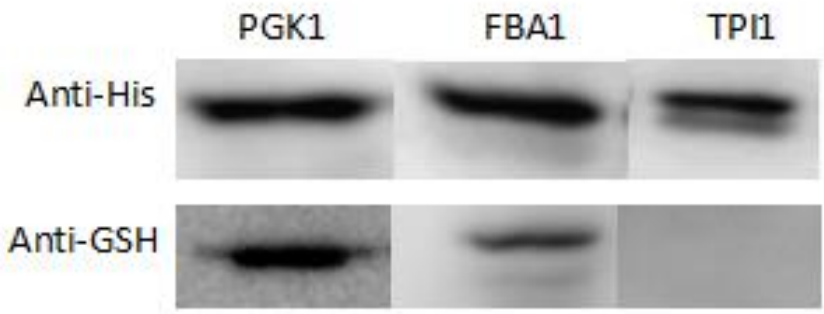


Figure3.4 Invivo glutathionylation analysis of proteins.Cell Lysates from H_2O_2 treated cells were purified using Ni-NTA beads .The blots were probed with mouse anti-His and mouse anti-GSH primary antibodies and goat anti-mouse HRP conjugated IgG as secondary antibody.The signal was detected using Luminata forte western HRP substrate.

3.4 Recombinant His-tagged Fba1,Pgk1 & Tpi1 Protein purified through Ni-NTA method and checked for glutathionylation

Cells expressing Pgk1p,Tpi1p and Fba1p were exposed to 2mM H_2O_2 for 20 min and were lysed and purified (see 2.11) with Ni-NTA beads for 3-4 hours at 4 ℃ .Protein was finally eluted in 60 µlelution buffer.Equal amount of samples were denatured and were resolved by SDS PAGE (12% acrylamide gel),electroblotted on PVDF membrane and was probed with mouse monoclonal anti-His and anti-GSH antibody.Finally , blots were probed with goat anti-mouse HRP conjugated secondary IgG and visualised using chemiluminescence detection reagent.

Protein expression was observed in both stressed and unstressed conditions.

In vivo glutathionylation under H_2O_2 stress was observed in case of PGK1 and FBA1 but not in TPI1 protein. Tpi1p is also known to get glutathionylated in vivo [74].

We checked the glutathionylation of Pgk1p, Fba1p and Tpi1p in stress vs non stress conditions.

3.5 Comparative glutathionylation in stress vs without stress cells.

Pgk1, Tpi1 and Fba1 overexpressing cells in exponential phase were dissolved in water and divided into three sets.One set was control without any stress(C), other two sets were given H2O2 stress of 20 min(Tests-T). After that one test (T)and control(C) were washed and freezed at -80 ℃.Other set of stressed cells(T) were washed and left for 2 hours in water/media to relieve stress.This set was called as Deglutathionylation in media/water(DM/DW).Then these cells were lysed and purified protein was checked for level of expression and glutathionylation in various conditions mentioned above.

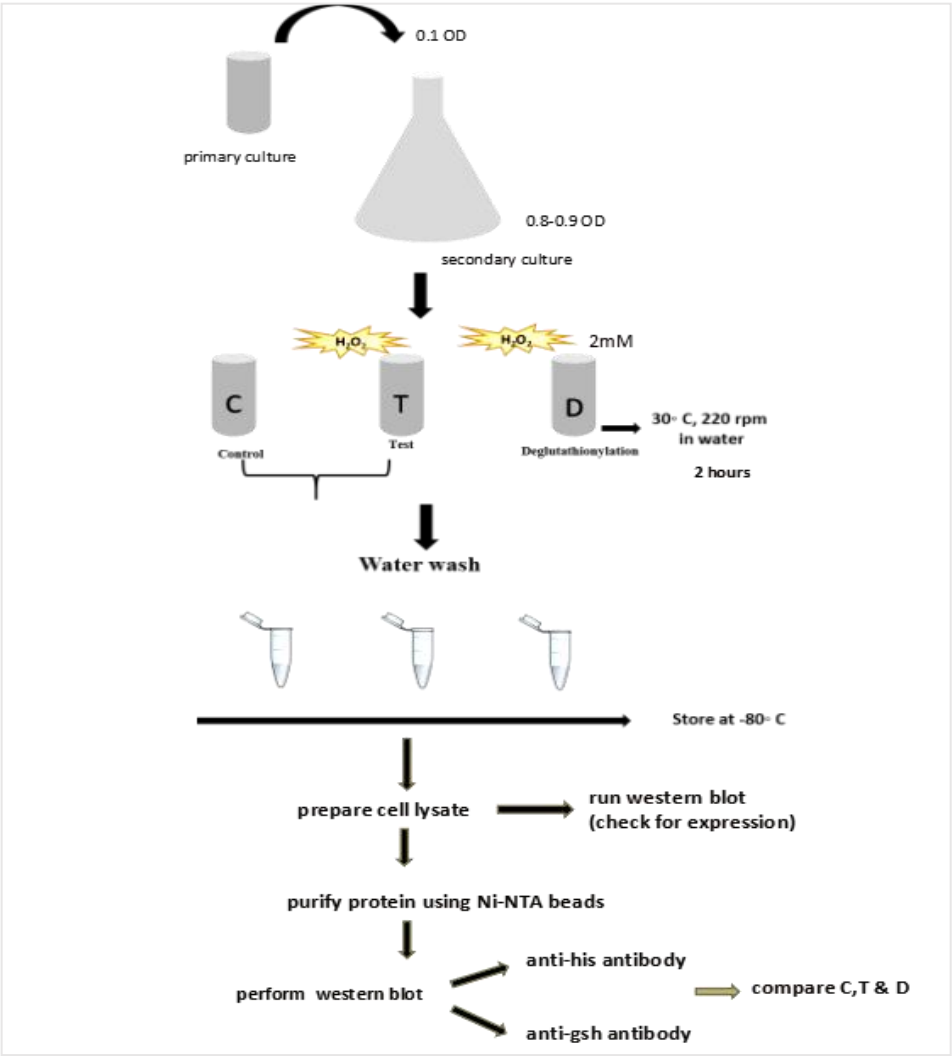
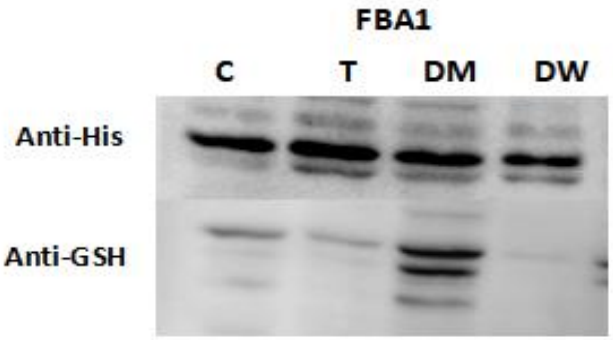
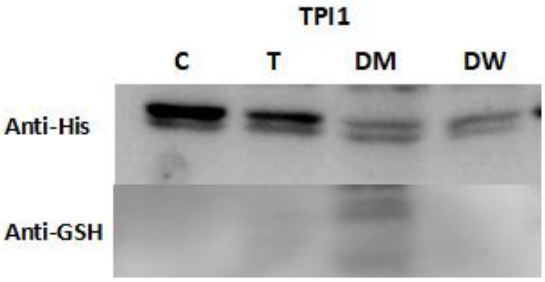


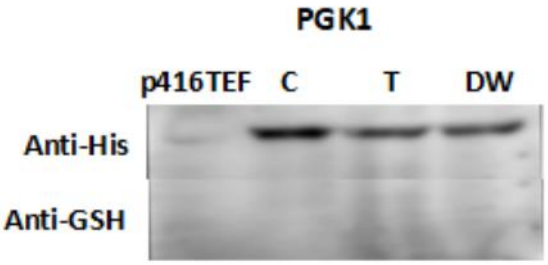
Figure3.5: Strategy to analyse the differential glutathionylation of glycolytic enzymes in stress vs non-stress conditions.



(a)Comparative glutathionylation of Fba1p under stress vs non-stress and after withdrawal of stress.



(b) Comparative glutathionylation of Tpi1p under stress vs non-stress and after withdrawal of stress



(c) Comparative glutathionylation of Pgk1p under stress vs non-stress and after withdrawal of stress

Figure3.6 Comparative glutathionylation of FBA1,TPI1 and PGK1 without oxidative stress,with oxidative stress and after the withdrawal of oxidative stress**.**Equal O.D. of cells were lysed to purify proteins and same sample amount was used for western blot.The blots were probed with mouse anti-His and mouse anti-GSH primary antibodies and goat anti-mouse HRP conjugated IgG as secondary antibody.The signal was detected using Luminata forte western HRP substrate.

Differential glutathionylation was seen in case of Fba1p.Fba1p showed comparable levelsof glutathionylation in control(without stress) as well as in test.However, both of them showed higher levels of glutathionylation compared to deglutathionylation in water.This suggests that Fba1p gets deglutathionylated in water once the stress is removed. We also observed glutathionylation in control (without stress) samples.

In case of Tpi, no glutathionylation was observed as was in the previous experiment.

In case of Pgk,we used the new antibody lot ,which didn't work.

Glutathionylation of proteins has been shown to occur either enzymatically or non-enzymatically[85]. Both these reactions can proceed via different pathways[81-85].In mammals, the proteins involved to catalyze glutathionylation are known to be either the glutathione S-transferases(belongingto the omega and pi family) or the glutaredoxins.

When we assesed the *S.cerevisiae* genome, There are three known reported glutathione S-transferases related to GST omega family .These are Gto1p, which is peroxisomal, and Gto2p and gto3p ,which are cytoplasmic[215].Two other proteins having a GST domain that does not group with mammalian GSTs,but are related to bacterial GSTs,are the Glutathione Transferases-Gtt1p and Gtt2p, known to localise to cytoplasm and endoplasmic reticulum[217].Finally,the glutaredoxins,Grx1p and Grx2p have also been reported to have glutathione S-transferase activity[216] .Monothiol Grx3,Grx4 and Grx5 could also be involed as Grx3 and Grx4 also show GST acitvity .

To examine if deglutathionylation of these proteins is enzyme-dependent, we investigated the possible candidate enzymes involved in the process.The thioredoxins, glutaredoxins and sulphiredoxins are possible candidate enzymes involved in deglutathionylation.We expressed fba1p and pgk1p under the different glutaredoxin(grx1 Δ, grx2 Δ grx3 Δ, grx4 Δ,grx5 Δ) and thioredoxins (trx1Δ and trx2Δ) mutant backgrounds and examined the glutathionylation status of the proteins after exposing the cells to oxdative stress .Since, we don't have any prior basis for the role of these proteins in glutathionylation of glycolytic enzmyes, we evaluated all of these for their role in glutathionylation/deglutathionylation.

3.6 Grx3 may be involved in Glutathionylation of Fba1p

To investigate the glutathionylating enzyme for Fba1p(Figure 3.6), Fba1p was expressed in glutaredoxins mutants. These mutants were checked for glutathionylation after the cells were

exposed to oxidative stress.All the deletions mutants showed comparable protein expression .Among, the different mutants,we observed that glutathionylation was specifically reduced in case of grx3Δ mutant as compared to other grx mutants.

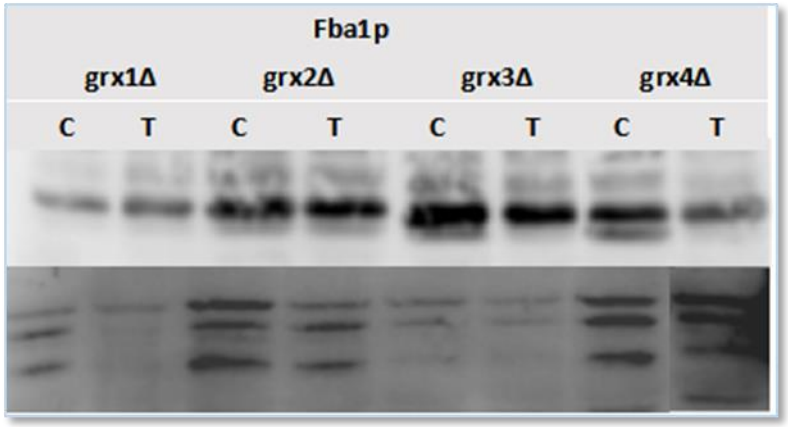


Figure3.6 Expression and glutathionylation of Fba1p in different glutaredoxin mutants Fba1p protein was purified from glutaredoxins grx1Δ,grx2Δ,grx3Δ and grx4Δ mutants after exposure to 2mM H_2O_2 for 20 min. C is control without H2O2 treatment and T is with H2O2 treatment.After washing cells were lysed using glass beads and His tagged fba1p was purifed .The purified protein was blotted and probed for his tag & glutathionylation.

3.7 Trx and Grx1 may be involved in Glutathionylation cycle of Pgk1p

We were also interested in investigating enzymes involved in the glutathionylation of Pgk1p, the enzymes involved in regulation.To begin with we checked the glutathionylation of Pgk1p in glutaredoxins ,Gtt and thioredoxin deletions background(grx1Δ, grx2Δ, gtt1Δ, gtt2Δ ,trx1Δ& trx2Δ).Equal O.D. of cells in exponential phase were given H_2O_2 stress for 20 min.Then the cells were lysed and expression and glutathionylation of Pgk1 was checked in crude lysate.In gtt1Δ ,gtt2Δ ,grx2Δ background,levels of glutathionylation were unaffected.

In grx1Δ,levels of glutathionylation of pgk1 was negligible.Also,Trx1 & Trx2 deletion mutants showed comparatively less levels of glutathionylation relative to their protein expression.But, surprisingly , trx2Δ mutants showed high levels of glutathionylation post stress removal.This may be because thioredoxins are known to decrease the glutathionylation of proteins once stress is over.So, Trx2 may be the deglutathionylating enzyme.Less amount of glutathionylation was found in grx1Δ, trx1Δ & trx2 Δ mutants. But which of this in involved in glutathionylation/deglutathionylation? To further confirm these results, we

purified the proteins from the cell lysate that were followed by probing with anti-GSH and anti-His antibody.

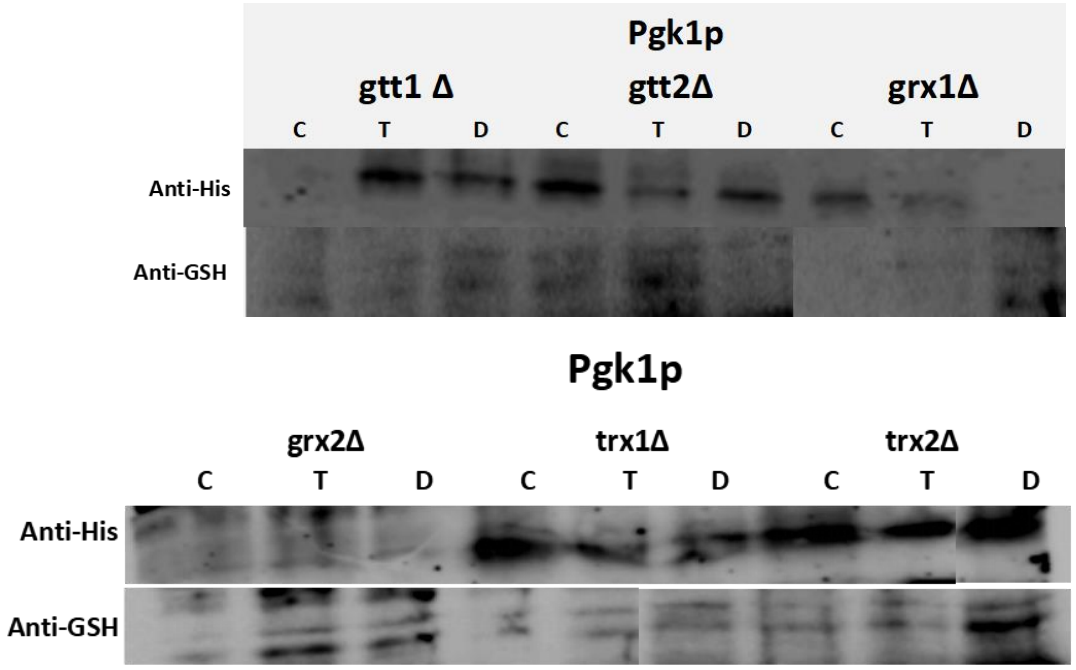


Figure:3.8 Expression and glutathionylation of Pgk1p in different mutant backgrounds.Cells overexpressing Pgk1p in various deletion backgrounds were treated with 2mM H_2O_2 for 20 min.After washing cells were lysed using glass beads and cell lysates were analyzed in western blot.The blots were probed with mouse anti-His and mouse anti-GSH primary antibodies and goat anti-mouse HRP conjugated IgG as secondary body.The signal was detected using Luminata forte western HRP substrate.

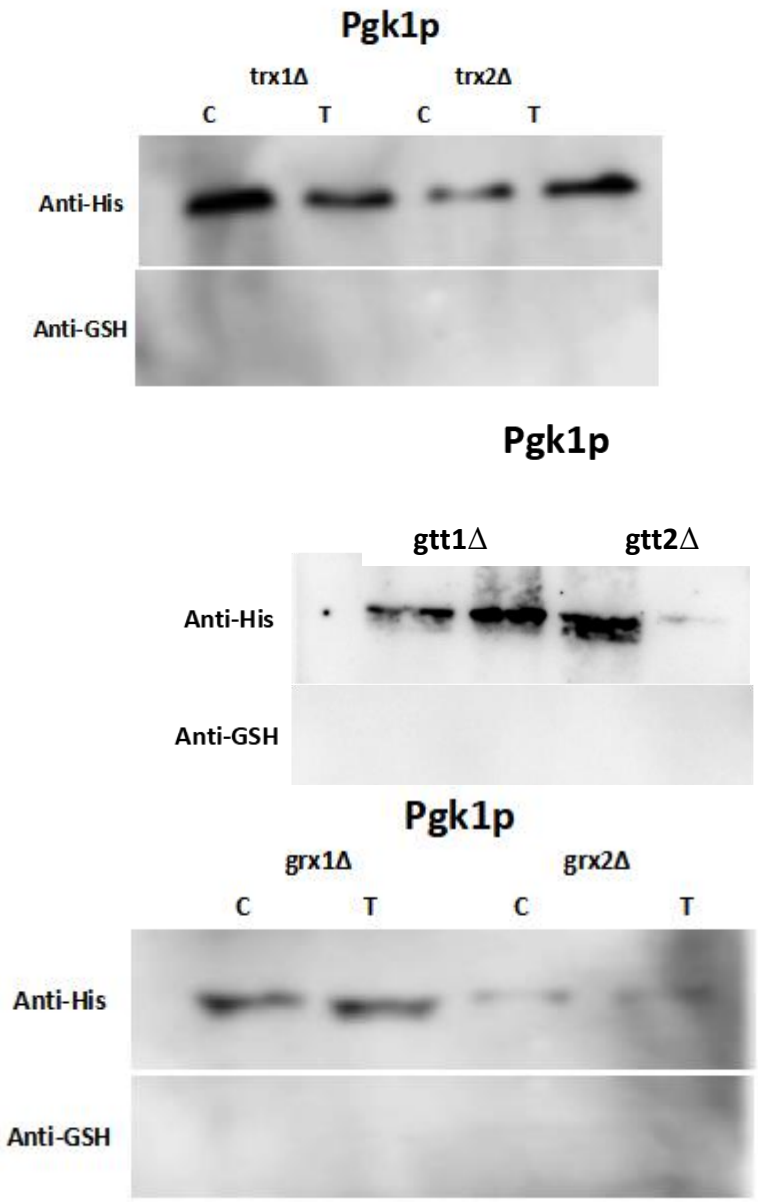


Figure3.9:Analysis of expression and glutathionylation of Pgk1p in various deletion mutants.PGK1 was overexpressed in trx1Δ,trx2Δ, grx1Δ, grx2,gtt1Δ& gtt2Δ background.Equal O.D. of cells were lysed using glass beads and his tagged Pgk1p was purified using Ni-NTA and same sample amount was used for western blot.The blots were probed with mouse anti-His and mouse anti-GSH primary antibodies and goat anti-mouse HRP conjugated IgG as secondary antibody.The signal was detected using Luminata forte western HRP substrate.C is control without H_2O_2 and T is test with H_2O_2stress.

Conclusion and Discussion

Glutathionylation is known to regulate the function of many proteins.During glycolysis, many proteins gets glutathionylated. In my thesis, I have looked into its enzymatic regulation by glutathionylation.For that, I overexpressed glycolytic enzymes in yeast in various deletion mutants and evaluated their glutathionylation.

I found that Fba1p and Pgk1p get glutathionylated in vivo.However, surprisingly I observed glutathionylation of both Fba1p and Pgk1p in stress as well as without stress.In fact, lesser glutathionylation was observed in H_2O_2 treated cells.In case of Pgk1p, previous studies have shown glutathionylation in basal conditions also [71].The possibility, therefore, exists that Pgk1p and Fba1p undergo glutathionylation in normal physiological conditions.

Fba1p glutathionylation decreased the grx3Δ mutant background. So, it is likely that grx3 may play a role in glutathionylation of Fba1.

Previous data have shown Fba1p activity is not affected due to glutathionylation. However, the previous study did not evaluate Fba1p activity in purified protein but in cell lysate and glutathionylation was not checked before and after stress [74].

Yeast Pgk1p glutathionylation was also evaluated .The glutathionylation of Pgk1p was diminished in grx1Δ cells, as observed in western blot. Also, Trx2 deletion caused rise in glutathionylation of Pgk1p,even when the cells were allowed to recover after H_2O_2stress.Increased glutathionylation of of Pgk1p even after removal of stress points to the role of Trx2 in deglutathionylation of Pgk1p.

The results described in this thesis are still very preliminary.The experiments need to be repeated several times to ensure that what we have observed is indeed true and reproducible.

However, the availability of the clones and the observed expression of at least three proteins would enable us to investigate the role of glutathionylation of glycolytic enzymes of yeast in oxidative stress in a more exhaustive manner.

References

1. Romano, AH; Conway, T (1996). "Evolution of carbohydrate metabolic pathways". Res Microbiol. 147 (6–7): 448–55.
2. Keller; Ralser; Turchyn (Apr 2014). "Non-enzymatic glycolysis and pentose phosphate pathway-like reactions in a plausible Archean ocean". Mol Syst Biol. 10 (4): 725.
3. Kim BH, Gadd GM. (2011) Bacterial Physiology and Metabolism, 3rd edition.
4. erg, J. M.; Tymoczko, J. L.; Stryer, L. (2007). Biochemistry (6th ed.). New York: Freeman. p. 622.
5. Rintala et al.,Transcription of hexose transporters of *Saccharomyces cerevisiae*is affected by change in oxygen provision BMC Microbiology20088:53
6. Boles E., Hollenberg C. P. (1997) The molecular genetics of hexose transport in yeasts. FEMS Microbiol. Rev.21:85–111.
7. Herrero P., Galindez J., Ruiz N., Martinez Campa C., Moreno F. (1995) Transcriptional regulation of the Saccharomyces cerevisiae HXK1, HXK2and GLK1 genes. Yeast 11:137–144.
8. Cárdenas M. L., Cornish-Bowden A., Ureta T. (1998) Evolution and regulatory role of the hexokinases. Biochim. Biophys. Acta 1401:242–264.
9. Carbohydrate Res. 1999 May 31;318(1-4):110-5.Competitive inhibitors of yeast phosphoglucose isomerase: synthesis and evaluation of new types of phosphorylated sugars from the synthon D-arabinolactone-5-phosphate.Hardré R1, Salmon L.
10. Inhibition of triosephosphate isomerase by phosphoenolpyruvate in the feedback-regulation of glycolysis Nana-Maria Grüning Dijun Du,1 Markus A. Keller, Ben F. Luisiand Markus Ralser
11. Sharma S, Mustafiz A, Singla-Pareek SL, Shankar Srivastava P, Sopory SK. Characterization of stress and methylglyoxal inducible triose phosphate isomerase (OscTPI) from rice. Plant Signal Behav. 2012;7(10):1337–1345. doi:10.4161/psb.21415

12. Krieger K and Ernst JF (1994) Iron regulation of triosephosphate isomerase transcript stability in the yeast Saccharomyces cerevisiae. Microbiology 140 (Pt 5):1079-84 PMID: 8025673

13. Scott EW and Baker HV (1993) Concerted action of the transcriptional activators REB1, RAP1, and GCR1 in the high-level expression of the glycolytic gene TPI. Mol Cell Biol 13(1):543-50 PMID: 8417350

14. Glycolysis : All Steps with Diagram, Enzymes, Products, Energy Yield and Significance(by Editorial Team on January 4, 2019 in Biochemistry) [https://laboratoryinfo.com/glycolysis-steps-diagram-energy-yield-and-significance]

15. SGD yeast pathways-*Saccharomyces cerevisiae* Pathway: glycolysis url: https://pathway.yeastgenome.org/YEAST/new-image?type=PATHWAY&object=GLYCOLYSIS

16. Delgado ML, et al. (2001) The glyceraldehyde-3-phosphate dehydrogenase polypeptides encoded by the Saccharomyces cerevisiae TDH1, TDH2 and TDH3 genes are also cell wall proteins. Microbiology 147(Pt 2):411-7

17. Europian Bioinformatics Institute :https://www.ebi.ac.uk/interpro/potm/2004_2/Page2.htm

18. Packham EA, et al. (1996) The multifunctional transcription factors Abf1p, Rap1p and Reb1p are required for full transcriptional activation of the chromosomal PGK gene in Saccharomyces cerevisiae. Mol Gen Genet 250(3):348-56 PMID: 8602150

19. Wu S, Storey JM, Storey KB. Phosphoglycerate kinase 1 expression responds to freezing, anoxia, and dehydration stresses in the freeze tolerant wood frog, Rana sylvatica. J Exp Zool A Ecol Genet Physiol. 2009 Jan 1;311(1):57-67. doi: 10.1002/jez.495. PMID:18785212 doi:http://dx.doi.org/10.1002/jez.495

20. Hogg PJ. Biological regulation through protein disulfide bond cleavage. Redox Rep. 2002;7(2):71-7. doi: 10.1179/135100002125000299. PMID:12189052

21. Entian KD, et al. (1987) Studies on the regulation of enolases and compartmentation of cytosolic enzymes in Saccharomyces cerevisiae. Biochim Biophys Acta 923(2):214-21

22. MOLECULAR AND CELLULAR BIOLOGY, Aug. 1987, p. 2753-2761.Transcription of the Constitutively Expressed Yeast Enolase Gene ENOJ Is Mediated by Positive and Negative cis-Acting Regulatory Sequences REGINA COHEN, TERESA YOKOI, JANICE P. HOLLAND, ALAN E. PEPPER, AND MICHAEL J. HOLLAND

23. Boles E, et al. (1997) Characterization of a glucose-repressed pyruvate kinase (Pyk2p) in Saccharomyces cerevisiae that is catalytically insensitive to fructose-1,6-bisphosphate. J Bacteriol 179(9):2987-93

24. Sprague GF (1977) Isolation and characterization of a Saccharomyces cerevisiae mutant deficient in pyruvate kinase activity. J Bacteriol 130(1):232-41 PMID: 323230

25. Fraenkel DG (2003) The top genes: on the distance from transcript to function in yeast glycolysis. Curr Opin Microbiol 6(2):198-201PMID: 12732312

26. Mck1, a Member of the Glycogen Synthase Kinase 3 Family of Protein Kinases, Is a Negative Regulator of Pyruvate Kinase in the Yeast Saccharomyces cerevisiae DERRICK T. BRAZILL, JEREMY THORNER, AND G. STEVEN MARTIN

27. Biochem. J. (2003) 374, 513–519 Protein S-thiolation targets glycolysis and protein synthesis in response to oxidative stress in the yeast Saccharomyces cerevisiae Daniel SHENTON and Chris M. GRANT

28. Innovative Medicine: Basic Research and Development: Diverting Glycolysis to Combat Oxidative Stress Edouard Mullarky and Lewis C. Cantley.

29. Glucose Signaling in Saccharomyces cerevisiae George M. Santangelo* Department of Biological Sciences, University of Southern Mississippi, Hattiesburg, Mississippi 39406-5018

30. Glucokinase: from Wikipedia, the free encyclopedia(http://test.daemon-secours-776655.com/index.php/Glucokinase)

31. M.A. Blázquez, R. Lagunas, C. Gancedo, J.M. GancedoTrehalose-6-phosphate, a new regulator of yeast glycolysis that inhibits hexokinases FEBS Lett., 329 (1993), pp. 51-54

32. https://www.wiley.com/college/pratt/0471393878/instructor/structure/phosphofructokinase/tutorial.html

33. The role of Glu187 in the regulation of phosphofructokinase by phosphoenolpyruvate

JMB Volume 235, Issue 1, 7 January 1994, Pages 68-72

34. CITRATE INHIBITION OF PHOSPHOFRUCTOKINASE AND THE PASTEUR EFFECT Maria L. Salas , E. Vifiuela , Margarita Salas 2 and A. Sols Department of Enzymology, fnstituto Centro de Investigaciones

35. //www.wiley.com/college/boyer/0470003790/structure/pfk/pfk_intro.htm.

36. Effect of pH on the Kinetics of Frog Muscle Phosphofructokinase" BAKULA TRIVEDI AND WILLIAM H. DANFORTH From the Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri

37. Activation by phosphate of yeast phosphofructokinase.
Banuelos M., Gancedo C., Gancedo J.M.J. Biol. Chem. 252:6394-6398(1977)

38. Stimulation of yeast phosphofructokinase activity by fructose 2,6-bisphosphate.Avigad G.Biochem. Biophys. Res. Commun. 102:985-991(1981)

39. A yeast phosphofructokinase insensitive to the allosteric activator fructose 2,6-bisphosphate. Glycolysis/metabolic regulation/allosteric control.
Heinisch J.J., Boles E., Timpel C.J. Biol. Chem. 271:15928-15933(1996)

40. Single point mutations in either gene encoding the subunits of the heterooctameric yeast phosphofructokinase abolish allosteric inhibition by ATP.Rodicio R., Strauss A., Heinisch J.J.J. Biol. Chem. 275:40952-40960(2000)

41. Novel sensing mechanisms and targets for the cAMP–protein kinase A pathway in the yeast Saccharomyces cerevisiae Johan M. Thevelein Johannes H. De Winde

42. Mck1, a Member of the Glycogen Synthase Kinase 3 Family of
Protein Kinases, Is a Negative Regulator of Pyruvate
Kinase in the Yeast Saccharomyces cerevisiae
DERRICK T. BRAZILL, JEREMY THORNER, AND G. STEVEN MARTIN

43. Saccharomyces cerevisiae pyruvate kinase Pyk1 is PKA phosphorylation substrate in vitro Małgorzata Cytryńska Magdalena Frajnt Teresa Jakubowicz

44. http://imed.stanford.edu/curriculum/session6/content/09-Regulation_of_glycolysis.pdf

45. Post-translational modifications on yeast carbon metabolism: Regulatory mechanisms beyond transcriptional control.DataBiochimica et biophysica acta, ISSN: 0006-3002, Vol: 1850, Issue: 4, Page: 620-7 , 2015

46. Oxidative Modification of Proteins: An Emerging Mechanism of Cell Signaling. Frontiers in Physiology 3(September):369 · September 2012

47. Free Radic Biol Med. 2007 Sep 15;43(6):883-98. Epub 2007 Jun 15.S-glutathionylation in protein redox regulation. Dalle-Donne I[1], Rossi R, Giustarini D, Colombo R, Milzani A.

48. Jones, D. P. Redefining oxidative stress. Antioxid. Redox Signal. 8:1865–1879; 2006.

49. Finkel, T.; Holbrook, N. J. Oxidants, oxidative stress and the biology of ageing. Nature 408:239–247; 2000

50. Redox Biol. 2013 Dec 19;2:123-39. doi: 10.1016/j.redox.2013.12.011. Collection 2014.Redox regulation of mitochondrial function with emphasis on cysteine oxidation reactions. Mailloux RJ, Jin X, Willmore WG.

51. Curr Opin Pharmacol. 2007 Aug;7(4):381-91. Epub 2007 Jul 26.

52. Mechanisms of reversible protein glutathionylation in redox signaling and oxidative stress. Gallogly MM**,** Mieyal JJ**.**

53. Oakley A (May 2011). "Glutathione transferases: a structural perspective". Drug MetabolismReviews. 43 (2):138–51. doi:10.3109/03602532.2011.558093. PMID 21428697.

54. Atkinson HJ, Babbitt PC (November 2009). "Glutathione transferases are structural and functional outliers in the thioredoxin fold". Biochemistry. 48 (46): 11108–16. doi:10.1021/bi901180v. PMC 2778357. PMID 19842715.

55. The role of glutathione S-transferase P in signaling pathways and S-glutathionylation in cancer.radical biology & medicine, ISSN: 1873-4596, Vol: 51, Issue: 2, Page: 299-313Publication Year2011

56. Hayes JD, Pulford DJ. The glutathione S-transferase gene family: Regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit Rev Biochem Mol Biol. 1995;30:445–600.

57. Garcerá A, et al. (2006) Saccharomyces cerevisiae cells have three Omega class glutathione S-transferases acting as 1-Cys thiol transferases. Biochem J 398(2):187-96 PMID: 16709151

58. Role of Yeast Glutaredoxins as Glutathione S-transferases*Emma J. Collinsonand Chris M. Grant

59. A Novel Membrane-bound Glutathione S-Transferase Functions in the Stationary Phase of the Yeast Saccharomyces cerevisiaeJae H. Choi, Willard Lou and Ales Vancura

60. Martin, J.L. Thioredoxin- a fold for all reasons. Structure, 1995, 3, 245-250.

61. Grant CM (2001) Role of the glutathione/glutaredoxin and thioredoxin systems in yeast growth and response to stress conditions. Mol Microbiol 39(3):533-41 PMID: 11169096

62. Outten CE and Culotta VC (2004) Alternative start sites in the Saccharomyces cerevisiae GLR1 gene are responsible for mitochondrial and cytosolic isoforms of glutathione reductase. J Biol Chem 279(9):7785-91 PMID: 14672937

63. Herrero, E., Belli, G., & Casas, C. (2010). Structural and Functional Diversity of Glutaredoxins in Yeast. Current Protein & Peptide Science, 11(8), 659–668.

64. Pedrajas J.R., Porras P., Martinez-Galisteo E., Padilla C.A., Miranda-Vizuete A., Barcena J.A.Two isoforms of Saccharomyces cerevisiae glutaredoxin 2 are expressed in vivo and localize to different subcellular compartments Biochem. J., 364 (2002), pp. 617-623

65. Molina, M. M., G. Bellí, M. A. de la Torre, M.-A. Rodríguez-Manzaneque, and E. Herrero. 2004. Nuclear monothiol glutaredoxins of Saccharomyces cerevisiae can function as mitochondrial glutaredoxins. J. Biol. Chem. 279: 51923–51930.

66. Shenton Daniel, Perrone Gabriel, Quinn Kathryn A, Dawes Ian W, Grant Chris M. Regulation of protein S-thiolation by glutaredoxin 5 in the yeast Saccharomyces cerevisiae. J Biol Chem. 2002 May 10;277(19):16853–16859.

67. Mesecke N., Mittler S., Eckers E., Herrmann J.M., Deponte M.Two novel monothiol glutaredoxins from Saccharomyces cerevisiae provide further insight into iron–sulfur

cluster binding, oligomerization, and enzymatic activity of glutaredoxins Biochemistry, 47 (2008), pp. 1452-1463

68. Garrido EO, Grant CM (2002) Role of thioredoxins in the response of Saccharomyces cerevisiae to oxidative stress induced by hydroperoxides. Mol Microbiol 43: 993–1003

69. Thioredoxins function as deglutathionylase enzymes in the yeast Saccharomyces cerevisiae Darren Greetham, Jill Vickerstaff, Daniel Shenton, Gabriel G Perrone, Ian W Dawes and Chris M Grant BMC Biochemistry2010

70.Ché S. Pillay, Hendrik S. Hofmeyr, Brett G. Olivier, Jacky L. Snoep, Johann M. Rohwer Enzymes or redox couples? The kinetics of thioredoxin and glutaredoxin reactions in a systems biology context Biochemical JournalJan 01, 2009,417(1)269-277;

71. Biochem J. 2017 Mar 15;474(7):1175-1193. Modulation of the specific glutathionylation of mitochondrial proteins in the yeast Saccharomyces cerevisiae under basal and stress conditions.Gergondey R, Garcia C, Marchand CH, Lemaire SD, Camadro JM, Auchère F.

72. An evolving understanding of the S-glutathionylation cycle in pathways of redox regulation Free Radical biology and medicine Zhi-wei Ye, Shweta Singh Danyelle M. Townsend, Kenneth D. Tew

73. Godon, C., Lagniel, G., Lee, J., Buhler, J.-M., Kieffer, S., Perrot, M., Boucherie, H., Toledano, M. B. and Labarre, J. (1998) The H2O2 stimulon in Saccharomyces cerevisiae.J. Biol. Chem. 273, 22480–22489

74. Biochem. J. (2003) 374, 513–519 Protein S-thiolation targets glycolysis and protein synthesis in response to oxidative stress in the yeast Saccharomyces cerevisiae Daniel SHENTON and Chris M. GRANT

75. Sambrook, J., Fritsch E.F. and Maniatis T. (1989) *Molecular cloning: A Laboratory Manual*. Cold Spring Harbor Press, Cold Spring Harbor New York.

76. https://med.nyu.edu/research/boeke-lab/resources-and-protocols/yeast-genetics-microarrays/protocols/genomic-dna-isolation-gbm