Glutamine metabolism and energy homeostasis during oxidative stress

by

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Abstract

When living organisms are exposed to oxidative stress, they are known to devise intricate mechanisms to counter reactive oxygen species (ROS). It is well established that ROS lead to the reduction in the activity of numerous tricarboxylic acid (TCA) cycle enzymes and impede O_2-dependent energy production. In this study we demonstrate an alternative metabolic pathway to adenosine triphosphate (ATP) synthesis when the microbe *Pseudomonas fluorescens* is challenged by H_2O_2.in a medium with glutamine as the sole source of carbon and nitrogen. Under oxidative stress the microbe utilized glutamine synthease (GS) to release a constant supply of energy locked in the amide bond of glutamine. When grown in presence of H_2O_2, the level of GS was higher in the stressed cultures compared to the control. The up-regulation of phospho-transfer enzymes such as acetate kinase (ACK), adenylate kinase (AK), and nucleoside diphosphate kinase (NDPK) are involved in maintaining ATP homeostasis in the oxidatively challenged cells. The increased amount of pyruvate phosphate dikinase (PPDK) and phosphoenol pyruvate carboxylase (PEPC) in stressed cells helped fuel the synthesis of ATP. The enhanced activities of isocitrate dehydrogenase-NAD dependent (ICDH-NAD) and glutamate dehydrogenase (GDH) also provided intermediate metabolites for energy generation. These metabolic reconfigurations may reveal crucial therapeutic tools against infectious microbes dependent on host glutamine for proliferation.

Keywords: Glutamine synthetase, ATP production, oxidative stress, metabolic networks.

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Dedication

I dedicate this work to my Father Saeed Aldarini and Mother Sareeia Mubark who surrounded me with their endless support, encouragement, love and prayers. They have been a great source of motivation and inspiration.

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List of Abbreviations

ATP	Adenosine triphosphate
PEP	Phosphoenol pyruvate
NADH	Reduced nicotinamide adenine dinucleotide
$FADH_2$	Reduced flavin adenine dinucleotide
TCA cycle	Tricarboxylic acid cycle
ETC	Electron transport chain
SLP	Substrate level phosphorylation
PK	Pyruvate kinase
PGK	Phosphoglycerate kinase
ASCT	Acetate succinate CoA transferase
SCS	Succinyl CoA synthetase
AMP	Adenosine monophosphate
PPDK	Pyruvate phosphate dikinase
CK	Creatine kinase

AK	Adenylate kinase
ROS	Reactive oxygen species
H_2O_2	Hydrogen peroxide
$^1O_2$	Singlet oxygen
•OH	Hydroxyl radical
$O_2^{\bullet-}$	superoxide
SOD	Superoxide dismutase
GPx	Glutathione peroxidase
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
ICDH-NADP	NADP-dependent isocitrate dehydrogenase
ME	Malic enzyme
GDH-NADP	NADP-dependent glutamate dehydrogenase
GLS	Glutaminase
HMPB	(4-hydroxymethyl-3-methoxyphenoxy)butanoic acid

GS	Glutamine synthetase
kDa	Kilodalton
rTCA cycle	Reverse tricarboxylic acid cycle
µM	Micromolar
ACK	Acetate kinase
NDPK	Nucleoside diphosphate kinase
BN-PAGE	Blue native polyacrylamide gel electrophoresis
HPLC	High performance liquid chromatography
ADP	Adenosine diphosphate
PEPS	Phosphoenol pyruvate synthase
Pi	Inorganic phosphate
PPi	Sodium pyrophosphate
mL	Milliliter
°C	Celsius
min	Minute

µg	Microgram
rpm	Revolutions per minute
h	Hour
N	Phenylalanine
CSB	Cell storage buffer
PMSF	Phenylmethylsulphonyl fluoride
mM	Millimolar
s	Second
PMS	phenazine methosufate
g/L	Gram per liter
INT	Iodonitrotetrazolium
NAD	Nicotinamide adenine dinucleotide (oxidized)
αKGDH	αketoglutarate dehydrogenase
LDH	Lactate dehydrogenase
MDH	Malate dehydrogenase

mg/mL	Milligram per milliliter
DCPIP	Dichloroindophenol
PC	Pyruvate carboxylase
CFE	Cell free extract
αKG	αKetoglutarate
ACN	Aconitase
Fe	Iron
NADK	Nicotinamide adenine dinucleotide kinase
NADS	Nicotinamide adenine dinucleotide synthetase
NADP	Nicotinamide adenine dinucleotide phosphate

CHAPTER 1: Introduction & Objectives

1.1. Introduction

1.1.1.Metabolism: the sum of all cellular reactions

Cellular metabolism is a set of life-sustaining biochemical reactions. It provides living organisms a variety of crucial metabolites required in the production of energy, in repairing damaged cells, in generating new cells, in detoxifying toxic chemicals, and in transporting core nutrients (Figure1.1-1).

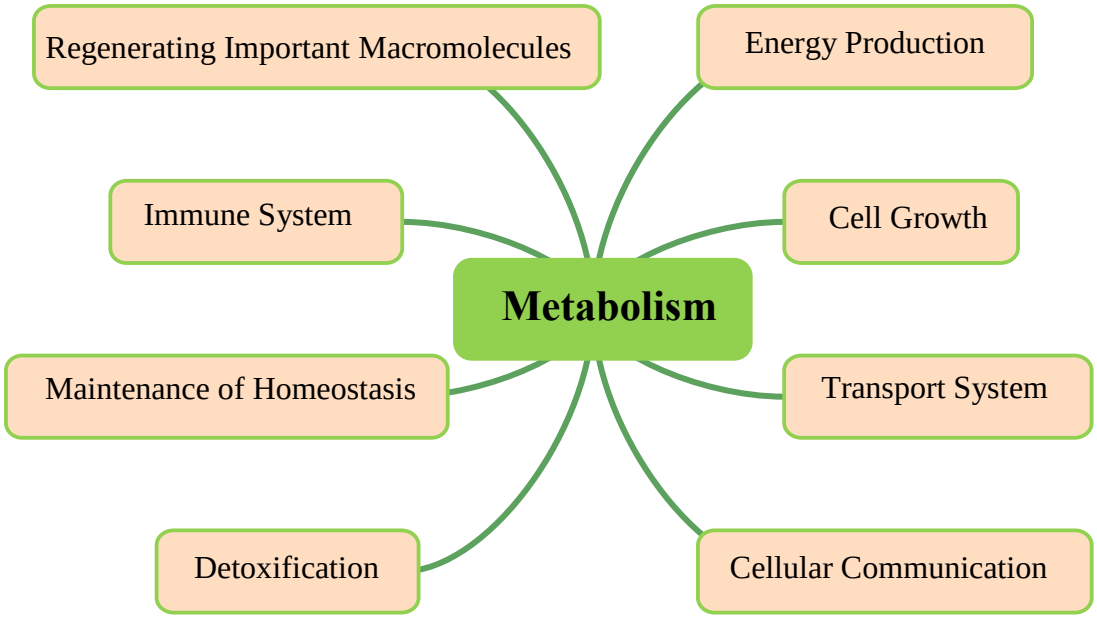

Figure 1.1-1: Major roles of metabolism in living systems

These metabolic features are catalyzed by enzymatic reactions that contribute to specific metabolic processes. Metabolism is highly regulated by sophisticated and inter-connected pathways that lead to unique metabolic networks (Rohmer et al. 2011). Metabolism can be broadly divided into catabolism and anabolism. Energy production pertains to catabolic reactions, which

degrade large molecules to liberate smaller molecules as well as energy. Living organisms utilize energy liberated from catabolism that subsequently participates in anabolic processes (Figure 1.1-2). The latter create new molecules that propel growth, reproduction and other crucial functions. (DeBerardinis and Thompson 2012). The main function of metabolism is to maintain the daily activities of all organisms. The metabolites produced are key precursors in the formation of proteins, lipids, carbohydrates, nucleic acids, and ATP, all of which are essential for the organism to survive (Tomàs-Gamisans et al. 2016)

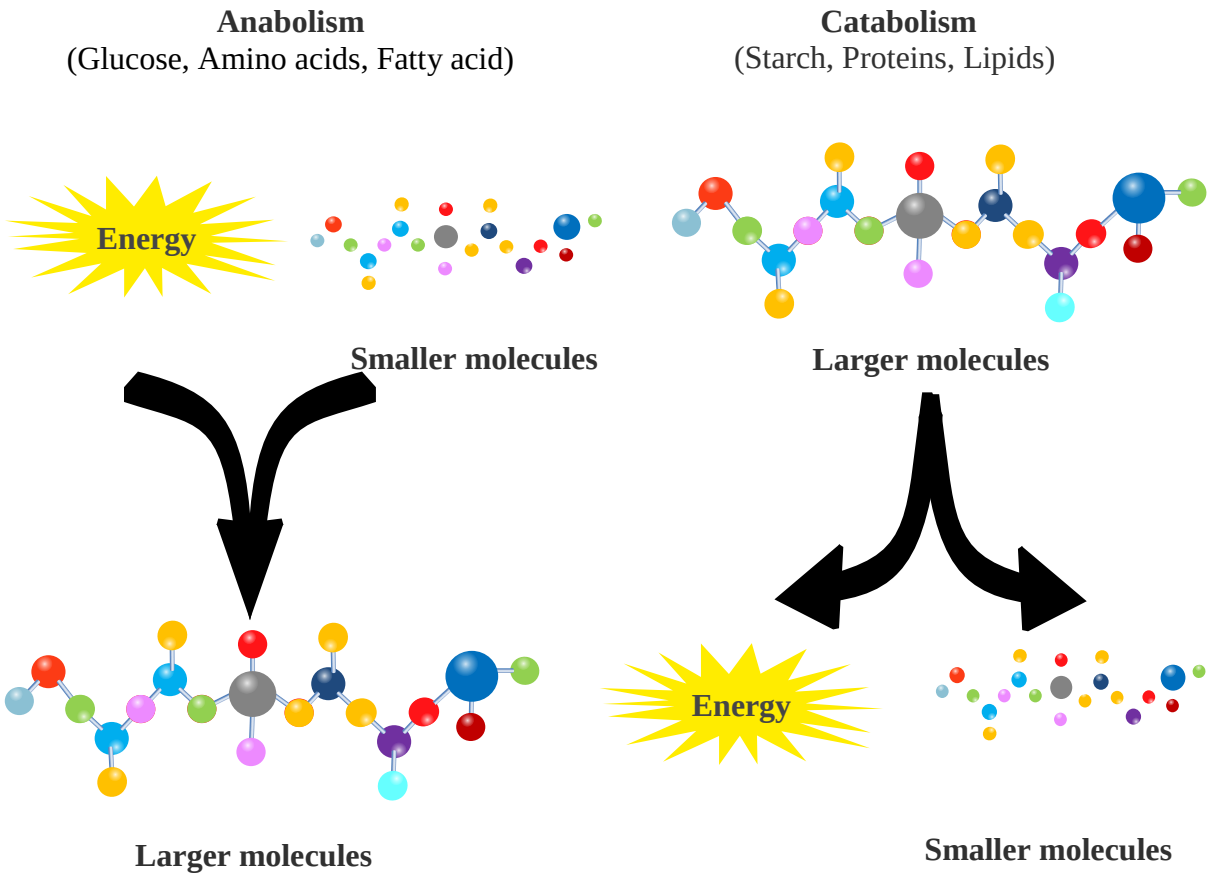

Figure 1.1-2: Catabolic and anabolic reactions (adapted from DeBerardinis and Thompson 2012)

Adenosine triphosphate (ATP) is the main chemical energy that drives directly or indirectly the inner workings of all known life-forms. ATP is the high-energy intermediate and energy currency molecule of all living cells. ATP contains one phoshoester bond bound by linkage of the α-phosphoryl group to 5′-oxygen of ribose and two phosphoanhydrides formed by the α,β and β,γ linkages between phosphoryl group (Figure 1.1-3). There are other high-energy or energy-rich phosphate that are also utilized as direct energy instead of ATP. These high energy compounds include enol phosphate such as phosphoenolpyruvate, acyl phosphates, and phosphoguanidines such as phosphocreatine. All these contain phosphate group-transfer potentials significantly greater than ATP (Table 1.1-1). Additionally, there are compounds with low energy phosphate such as glucose-6-phosphate or glycerol-3-phosphate that may contribute to the energy budget of the cell (Reddy and Wendisch 2014; Kammermeier 1987).

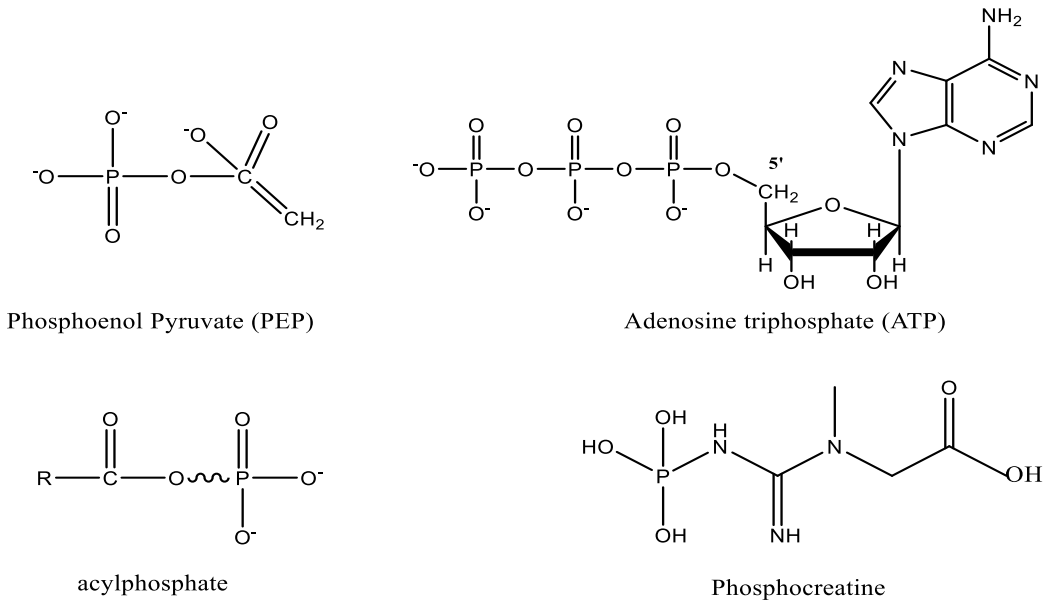

Figure 1.1-3: Chemical structure of adenosine triphosphate (ATP), Phosphoenol Pyruvate, Phosphocreatine, Acylphosphate

Table 1.1-1: High and low energy phosphate compounds and standard free energy values. (adapted from Carraro et al. 2014)

	$\Delta G^{\circ\prime}$ KJ mol^{-1}	Metabolite
High Energy	– 62	Phosphoenolpyruvate
	– 49	1,3–*Bis*phosphoglycerate
	– 43	phosphocreatine
	– 32	ATP
Low Energy	– 21	Glucose 1-phosphate
	– 14	Glucose 6-phoshate
	– 9	Glycerol 3-phoshate

1.1.2.Energy production mechanisms

There are many different chemical reactions that contribute to the formation of ATP in the cells. Most of the ATP in cells is made using the chemical energy from glucose or other reduced carbon source. Glucose can be synthesized via photosynthesis where the energy stored in the sunlight is trapped in the presence of CO_2 and water. This process can also result in the formation of organic molecules such as sugars, proteins, and lipids (Rosenberg et al. 2008). In prokaryotic cells photosynthesis and cellular respiration occur within cytoplasm and on the inner surfaces of the membrane while in eukaryotic cells these chemical processes are carried out in specific sites in the

organelles. Mitochondria are the site for cellular respiration. Chloroplasts are the main organelle for photosynthesis. (Kalaidzidis et al. 2001).

1.1.2.1. ATP Synthesis via oxidative phosphorylation

Oxidative phosphorylation is one of the most important metabolic module that is utilized by aerobic organisms to produce energy. During this process some biomolecules are oxidized in order to generate (ATP). The metabolic pathway is designed to liberate the greatest amount of (ATP) in most prokaryotic and eukaryotic systems. Initially, reducing factors like NADH and $FADH_2$ are released from the numerous carbon sources. In fact, the tricarboxylic acid (TCA) cycle is primarily programmed to generate three NADH and one $FADH_2$ from acetyl CoA with the concomitant liberation of 2 CO_2 (Figure 1.1-4). Tricarboxylic acid cycle is a set of enzyme-controlled reactions that occur within the mitochondria of all aerobic eukaryotic cells and the cytoplasmic membrane of prokaryotes (Cavalcanti et al. 2014). When the pyruvate usually released from glycolysis enters this metabolic network, it is converted into acetyl CoA. These acetyl CoA molecules are subsequently squeezed of their electrons with the aid of oxaloacetate. The latter is regenerated at the end of the cycle to further accompany the degradation of another acetyl CoA. The electrons captured in NADH and $FADH_2$ are transported via the electron transport chain (ETC). During the sequences of oxidation-reduction and the movement of electrons, a proton gradient and a membrane potential are created. The transport of e^- among the various complexes helps pump protons from one side of the membrane to the other. This results in a high concentration of protons on one side of the membrane and creates a proton gradient. These protons subsequently flow down the concentration gradient and are tapped into ATP by ATP synthase. This process is called aerobic respiration, as oxygen molecules are the ultimate acceptors of the released electrons

(Fernie et al. 2004; Nazaret et al. 2009; Van Dongen et al. 2011). In situations where oxygen is absent NADH and $FADH_2$ follow an alternative electron transport route.

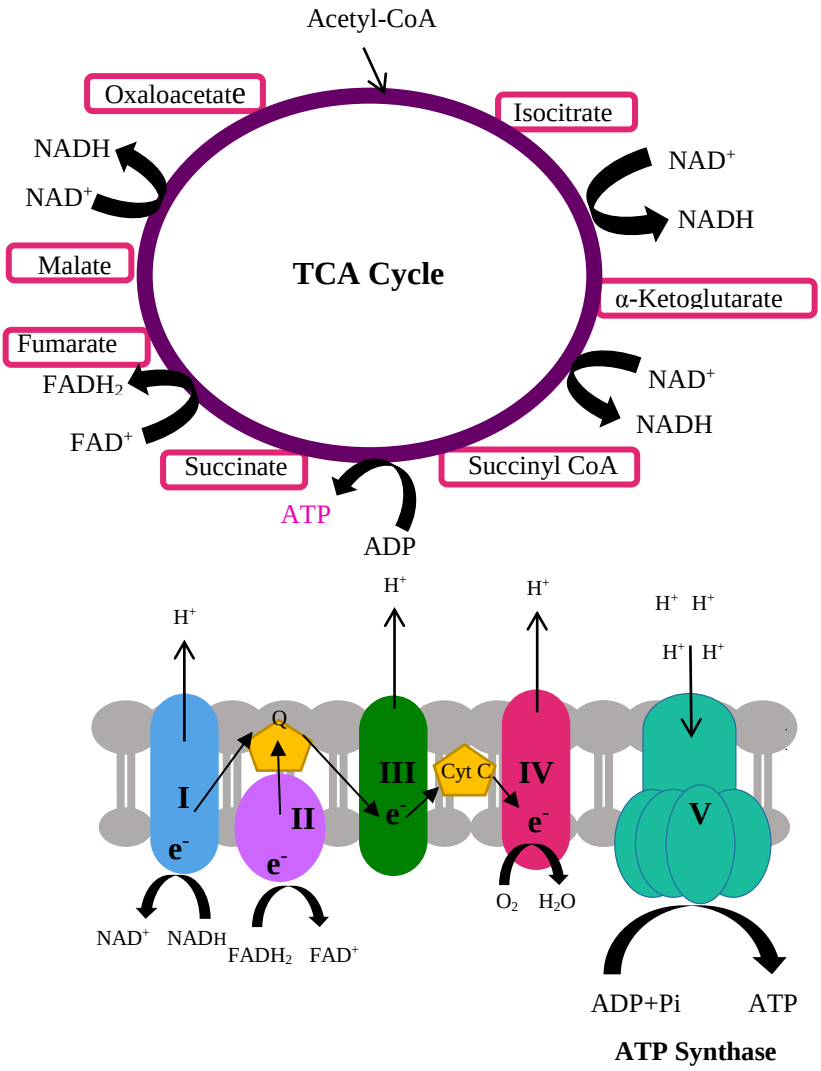

Figure 1.1-4: Tricarboxylic acid cycle and electron transfer chain (adapted from Van Dongen et al. 2011)

Many bacteria, archaea and several eukaryotes operate their electron transport chain with alternative or external electron acceptor when the oxygen concentration is low. The major alternative electron acceptors are nitrate (NO^{-}_{3}) sulphate (SO_4^{-2}), and some organic compounds such as fumarate. The microbe utilizes these electron acceptors depending on the microbial community and environmental conditions. As a result, these electron acceptors function as proton pumps (Figure 1.1-5) (van Niftrik et al. 2012; Kraftet et al. 2011; Ju et al. 2005). As electrons are transported through the ETC, about 2-5% of all oxygen is not completely reduced due to the electron leakage that leads to toxic (ROS) (Cadenas et al. 2000; Turrens et al. 2003).

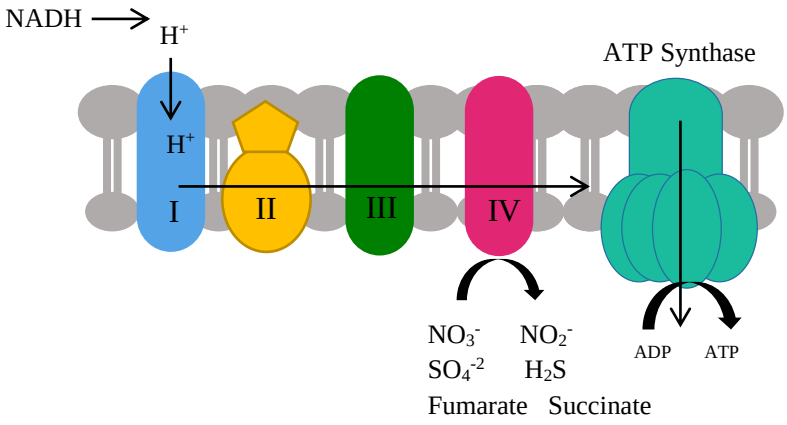

Figure 1.1-5: Electron transport chain with alternative electron acceptor A) nitrate, B) sulphate, C) fumarate

Production of the oxygen-dependent energy necessitates the presence of magnesium. Magnesium ion is a pivotal cofactor in stabilizing and maintaining ATP in living systems. Deficiency of magnesium contributes to the impediment in generating ATP during oxidative phosphorylation (Agarwal et al. 2014; Volpe et al. 2013; Vormann et al. 2003). Deficiency of oxygen, abnormalities in the TCA cycle and the ETC force organisms to rely on alternative pathway like substrate level phosphorylation to supplement their need in ATP (Bailey-Serres et al. 2010).

1.1.2.2. Substrate Level Phosphorylation and ATP Homeostasis:

In the absence of oxygen, most microorganisms rely on substrate level phosphorylation (SLP) to fulfill their energy need. Glycolysis is the crucial metabolic network that can generate ATP via SLP in living organisms. Pyruvate kinase (PK) and phophoglycerate kinase (PGK) are two glycolytic enzymes that mediate the formation of ATP during anaerobic respiration (Van Weelden et al. 2005). Many protists, including parasitic *helminths*, *amoeba*, *diplomonads*, *trichomonads*, and *trypanosomatids* extract as much energy as possible from various substrates via SLP. These eukaryotes, invoke an acetate succinate CoA transferase (ASCT) and succinyl CoA synthetase (SCS) cycle to generate ATP. The former is responsible for transferring the CoA from acetyl CoA to succinate and the energy is then preserved in the thioester bond of succinyl CoA (Bochud-Allemann et al. 2002). The succinyl CoA is then utilized to phosphorylate ADP to ATP. Furthermore, energy in the form of ATP can be obtained by the production of a high energy biomolecule known as phosphoenol pyruvate (PEP). This rich energy biomolecule is the main driver that fuels the phosphotransferase enzymes to generate maximum amount of energy. In this instance, pyruvate phosphate dikinase (PPDK) is able to fix AMP into ATP. ATP delivery can be facilitated through creatine kinase (CK), and adenylate kinase (AK) (Bringaud et al. 2010; Dzeja

et al. 2003; Radolf et al. 2016). In addition, creatine kinase (CK) is highly regulated in muscle cells and it plays a central role in phosphocreatine shuttle to transfer the high-energy phosphate from mitochondria to myofibrils in contracting muscle (figure 1.1-6) (Hettling et al. 2011). In some microbial systems, oxalyl CoA that is generated by a modified glyoxylate shunt, can power succinate into succinyl CoA. The latter then becomes a potent generator of ATP (Singh et al. 2009). In fact, under oxidative stress, *Pseudomonas fluorescens* is known to utilize this alternative ATP-generating strategy that obviates the need for O_2 (Alhasawi et al. 2015) [b] Although SLP generates less ATP, it has the advantage of not producing the toxic ROS unlike oxidative phosphorylation. The major drawback of the latter metabolic pathway is the formation of ROS that become a potent liability to the organisms (Flamholz et al. 2013).

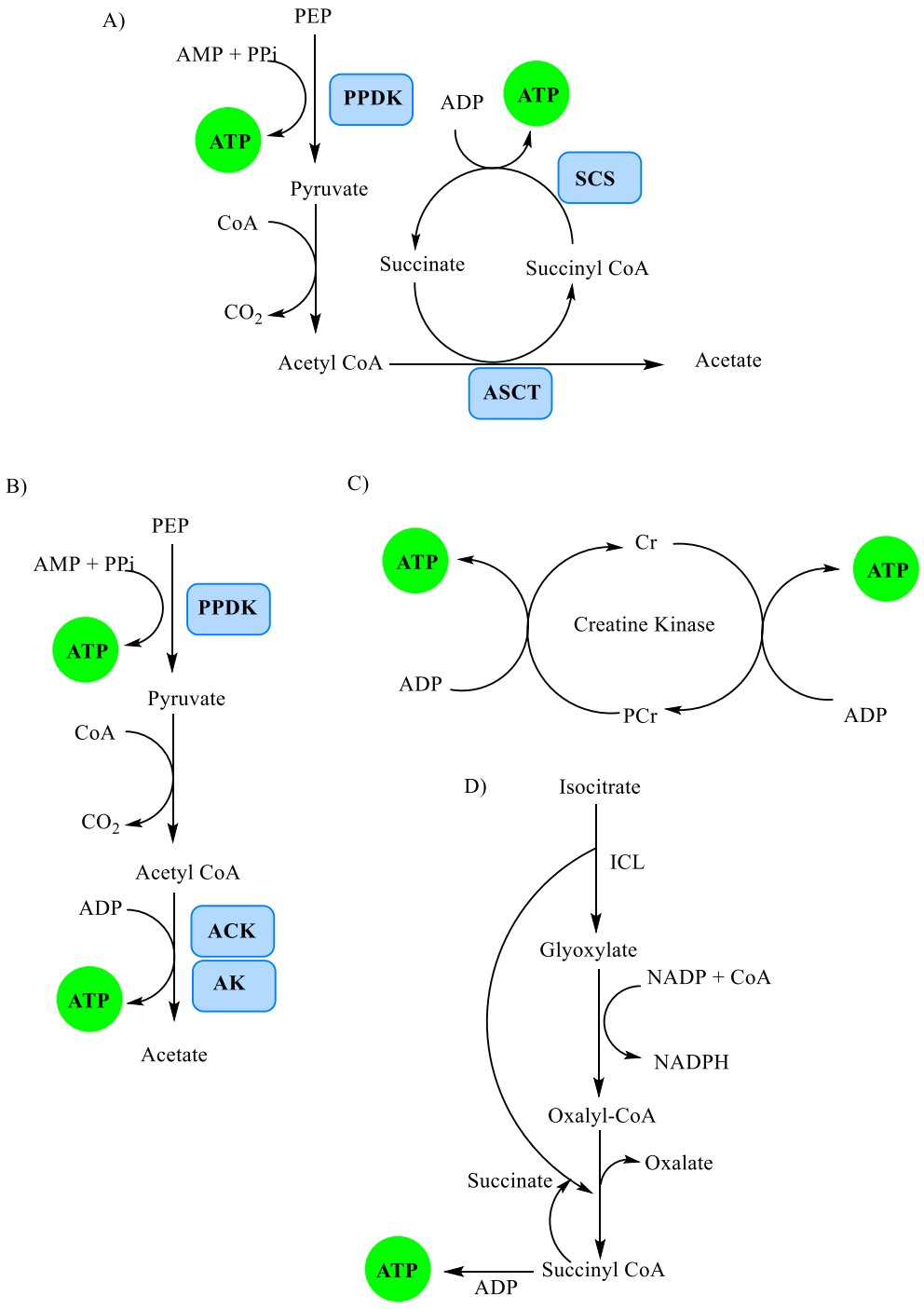

Figure 1.1-6: Substrate level phosphorylation powered by different metabolic networks. A) pyruvate orthophosphate dikinase (PPDK), succinyl CoA synthetase (SCS), and acetate succinate CoA transferase (ASCT) network. B) acetate kinase (ACK) and adenylate kinase (AK) network. C) creatine kinase (CK) in muscles. D) oxalyl CoA succinate transferase network.

1.1.3.Reactive oxygen species and anti-oxidative defence:

Reactive oxygen species (ROS) are a family of chemicals containing highly reactive free oxygen moieties that are generated during the reduction of oxygen as part of aerobic respiration (Wang et al. 2016). High concentrations of ROS are known to be harmful to all cells. However, in low concentrations ROS play very important physiological roles in gene expression, defense against infection and cellular growth. An excessive production of ROS may result in negative impact on numerous cellular constituents as proteins, carbohydrates, lipids and nucleic acids (Pande et al. 2015). ROS can be neutral molecules such as hydrogen peroxide H_2O_2, singlet oxygen $^1O_2$, or radicals such as hydroxyl radicals •OH, or ions such as superoxide $O_2^{\bullet-}$. The formation of ROS can cascade from one species to another (Figure 1.1-7) (Briege et al. 2012). The appearance of rich oxygen atmosphere on earth has led to the development of defense mechanisms to combat a high concentration of ROS and keep the ROS at acceptable levels. This process is referred to as antioxidant defense.

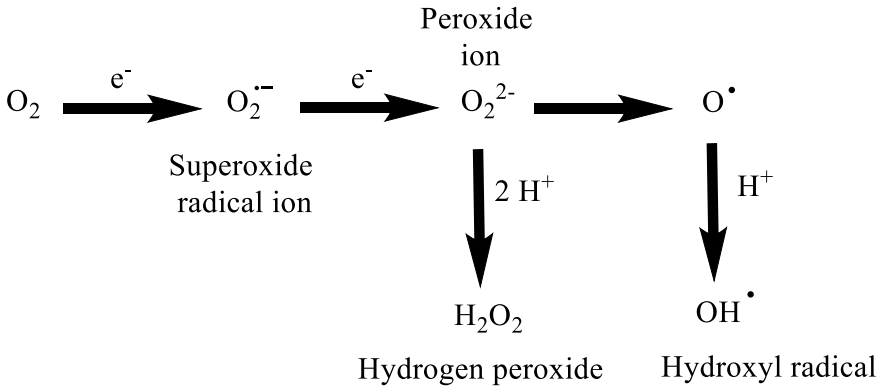

Figure 1.1-7: Production of ROS (adapted from Bhattacharya et al. 2015)

Living organisms have evolved a number of intricate processes to help maintain their survival against oxidative stress. Superoxide dismutase (SOD), glutathione, and catalase are the major enzymatic antioxidants (Agyei et al. 2015). Superoxide dismutase (SOD) is a metal-dependent enzyme. These metal ions are critical cofactors in the detoxification of ROS. SODs enzymes are able to neutralize superoxide $O_2^{\bullet-}$ and catalyze its dismutation to O_2 and H_2O_2 (Apel et al. 2004). Glutathione is a metabolite that mediates the neutralization of H_2O_2 with the aid of glutathione peroxidase(GPx). These biomolecules work in tandem to remove H_2O_2 by reducing it. They also oxidize lipids to water and lipid alcohol (Espinoza et al. 2008). Catalase is an iron-containing enzyme that detoxifies H_2O_2 by catalyzing the reaction between two hydrogen peroxide leading to the formation of water and O_2 (Figure 1.1-8) (Paravicini et al 2008; Bhattacharya et al. 2015).

$$2\ O_2^{\bullet-} + 2\ H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

$$2\ H_2O_2 \xrightarrow{CAT} 2\ H_2O + O_2$$

$$H_2O_2 + 2\ GSH \xrightarrow{GPx} 2\ H_2O + GSSG$$

Figure 1.1-8: Radical scavenging activity of super dismutase SOD, catalase CAT, and glutathione peroxidase GPx

There are also non-enzymatic or low molecular weight molecules that scavenge the radicals and render them inactive. In this category, the main non-enzymatic antioxidants are vitamin C and vitamin E. Vitamin E is known as α-tocopherol and it is a major antioxidant that is lipid-soluble. It acts as a powerful chain breaker. Vitamin E helps to intercept peroxyl radical chain reactions which result in lipid peroxidation. Vitamin C, also known as ascorbic acid is a water-soluble free radical scavenger. It reacts with free radical to generate ascorbyl radical, then interacts with polyunsaturated fatty acid or phospholipid peroxyl radical to form non-radical product. This reaction helps to break the chain reactions of lipid peroxidation (Nimse et al. 2015).

These antioxidants play significant role in allowing organisms to survive in an aerobic environment. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) is a powerful reducing agent that fuels the activity of most anti-oxidant. Therefore, the ATP production by oxidative phosphorylation cannot be performed effectively in the absence of a constant supply of NADPH (Singh et al. 2007). NADPH is the vital component in anti-oxidative defense mechanisms of all aerobic cells. NADPH producing enzymes are very active under oxidative environment. Glucose-6-phosphate dehydrogenase (G6PDH), NADP-dependent isocitrate dehydrogenase (NADP-ICDH), malic enzyme (ME), and NADP-dependent glutamate dehydrogenase (NADP-GDH) are the main enzymes as earmarked to produce NADPH (Mailloux et al. 2010). During oxidative stress, NAD is converted into NADP with the aid of NAD kinase. This metabolic adaptation helps in increasing NADPH production and decreasing NADH synthesis. The latter is a pro-oxidant (Alhasawi et al. 2015) [a] (Figure 1.1-9). Ketoacids are also known to detoxify (ROS) with concomitant formation of carboxylic acids and CO_2. Metabolic networks dedicated to the

production of pyruvate, glyoxylate and α ketoglutarate are enhanced during oxidative stress (Alhasawi et al. 2015 [a, b]; Thomas et al. 2016; Lemire et al.2010)

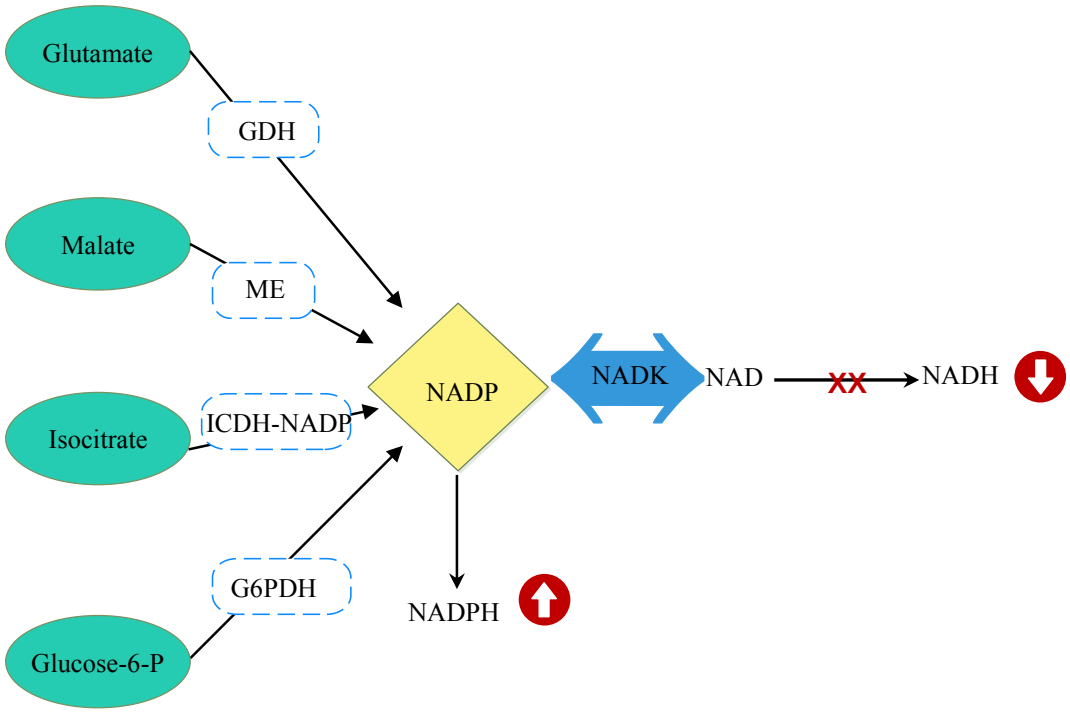

Figure 1.1-9: NADPH production and reduction in NADH synthesis are key to ROS survival. Glutamate dehydrogenase (GDH), malic enzyme (ME), NADP-dependent isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase (G6PDH), nicotinamide adenine dinucleotide phosphate (NADP), reduced nicotinamide adenine dinucleotide phosphate (NADPH), nicotinamide adenine dinucleotide kinase (NADK).

1.1.4.Glutamine metabolism:

Glutamine is the most abundant free amino acid in humans, making up to about 20% of free amino acid in the blood and 40% in muscles (Taylor et al. 2014). Glutamine belongs to a family of amino acid that is conditionally an essential amino acid. Hence, synthesis of glutamine occurs in most tissues during periods of rapid growth, stresses or illness. Glutamine has two nitrogen side chains (amide and amino groups). Therefore, it becomes a precursor for the biosynthesis of many nitrogen-containing compounds and is an essential intermediate in numerous metabolic pathways in the cell (Figure 1.1-10). Furthermore, glutamine acts as a nitrogen shuttle which serves as a vehicle for transporting and circulating ammonia in a non-toxic form within organs and is converted to ammonium or urea (Newsholme et al. 2003; Smith et al. 1990).

In cancer cells, glutamine is avidly used and consumed as an energy-producing substrate and a source of carbon and nitrogen for biomass accumulation. When glutamine enters the cells, it is converted by glutaminase (GLS) into glutamate and ammonia. Glutamate then is converted to α-ketoglutarate by glutamate dehydrogenase (GDH) which then contributes in the generation of ATP through the production of NADH and $FADH_2$, α-ketoglutarate can also be fixed into isocitrate by the reversible ICDH that is common in cancer cells (Figure 1.1-11). This metabolic pathway allows these cells to proliferate and thrive (Altman at el. 2016). Glutamine synthetase (GS) which also produces glutamate and ammonia in the presence of a nucleotide, plays particularly a significant role in nitrogen metabolism and is a vital ingredient for protein, and nucleic acid syntheses (DeBerardinis and Cheng 2010). However, GLS is more prominent during the catabolism of glutamine. GS in most bacteria and archaea is a multimeric enzyme containing 12 identical subunits with molecular masses ranging from 50 to 55 kDa each (Robinson et al. 2001).

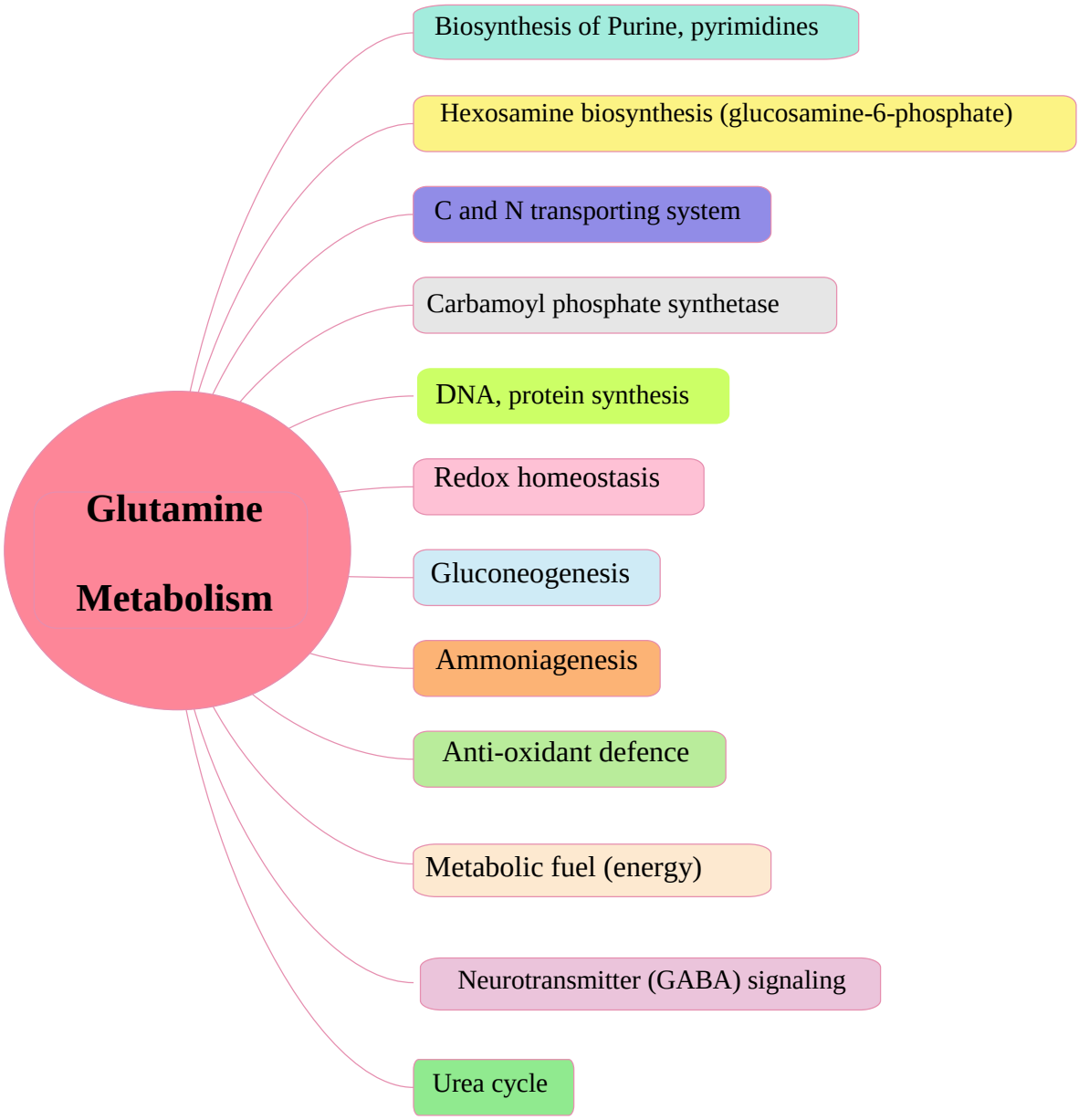

Figure 1.1-10: Overview of glutamine metabolism in mammalian cell (adapted from Newsholme et al. 2003; Smith et al. 1990)

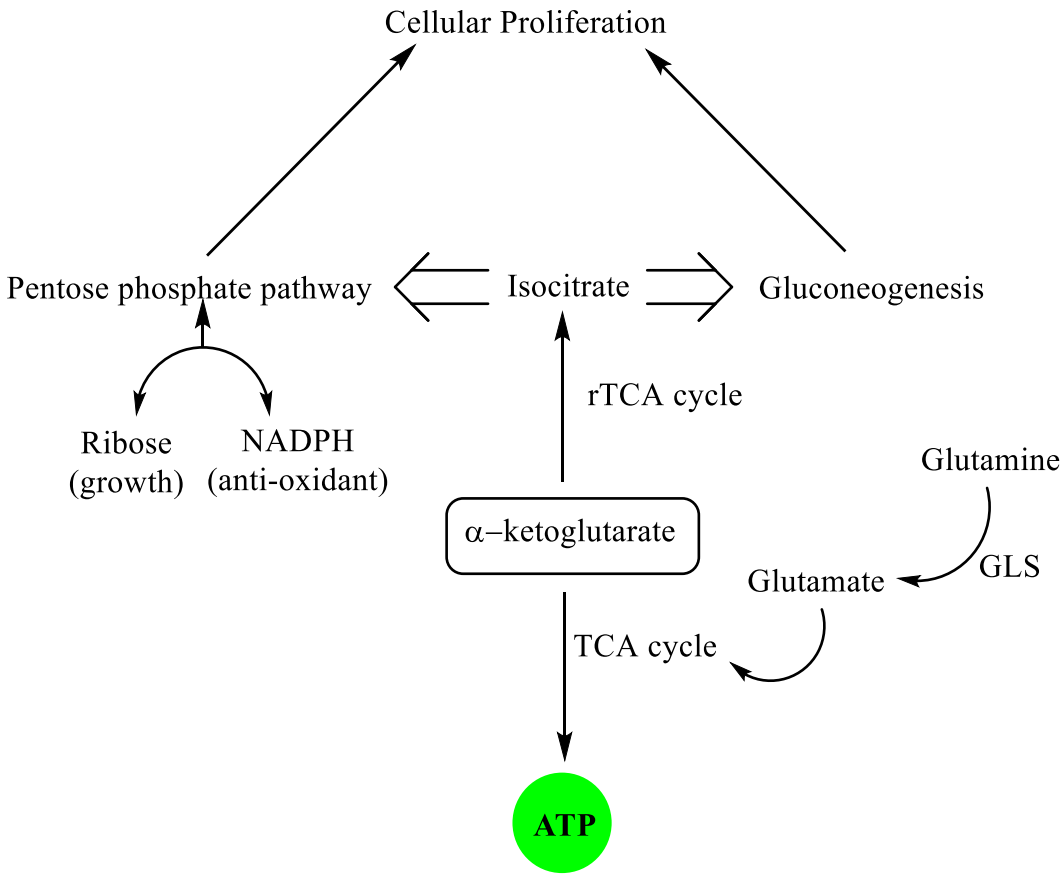

Figure 1.1-11: Glutamine catabolism in dividing cells. (rTCA) reverse tricarboxylic acid cycle, (TCA) tricarboxylic acid cycle

1.1.5.Research objectives

Although most of the literature is replete with information documenting how organisms detoxify ROS, it is important to evaluate the processes that allow these organisms to produce ATP under these ROS-rich conditions. ROS are known to severely perturb oxidative phosphorylation. *Pseudomonas fluorescens* is an excellent model system for studying these processes due to its flexibility in adapting to a variety of carbon sources and numerous toxic environmental stresses. It is a gram-negative, non-pathogenic microbe that grows in disparate ecological niches including the soil. It has the ability to grow rapidly in different media and is nutritionally versatile. Due to these unique biochemical attributes *P. fluorescens* is an excellent organism to study molecular adaptation in response to oxidative stress. In this instance the ability of the organisms to survive and maintain its ATP budget in a mineral medium with glutamine as the sole carbon and nitrogen source is evaluated. As the production of the glutamine necessitates energy, it is important to elucidate if the ATP locked in amide bond can be released during oxidative stress when the oxidative phosphorylation is severely compromised by (H_2O_2) challenge. This ROS (500 µM) has been shown to markedly compromise aerobic ATP production (Alhasawi el al. 2015) [a].

Glutamine synthetase (GS) that can mediate the degradation of glutamate with the concomitant production of ATP has been studied. The expression of its activity under H_2O_2 stress has been delineated. Furthermore, the role of phosphotransfer enzymes like acetate kinase (ACK) and nucleoside diphosphate kinase (NDPK) that may contribute to the ATP budget has also been monitored. Functional metabolome and proteome responsible for ATP synthesis in the presence of H_2O_2 challenge have also been assessed with the aid of blue native polyacrylamide gel electrophoresis (BN-PAGE), and high performance liquid chromatography (HPLC) (Figure 1.1-12). The findings on

these metabolic networks may reveal protective and therapeutic cues to control infectious microorganisms which depend on glutamine for their proliferation.

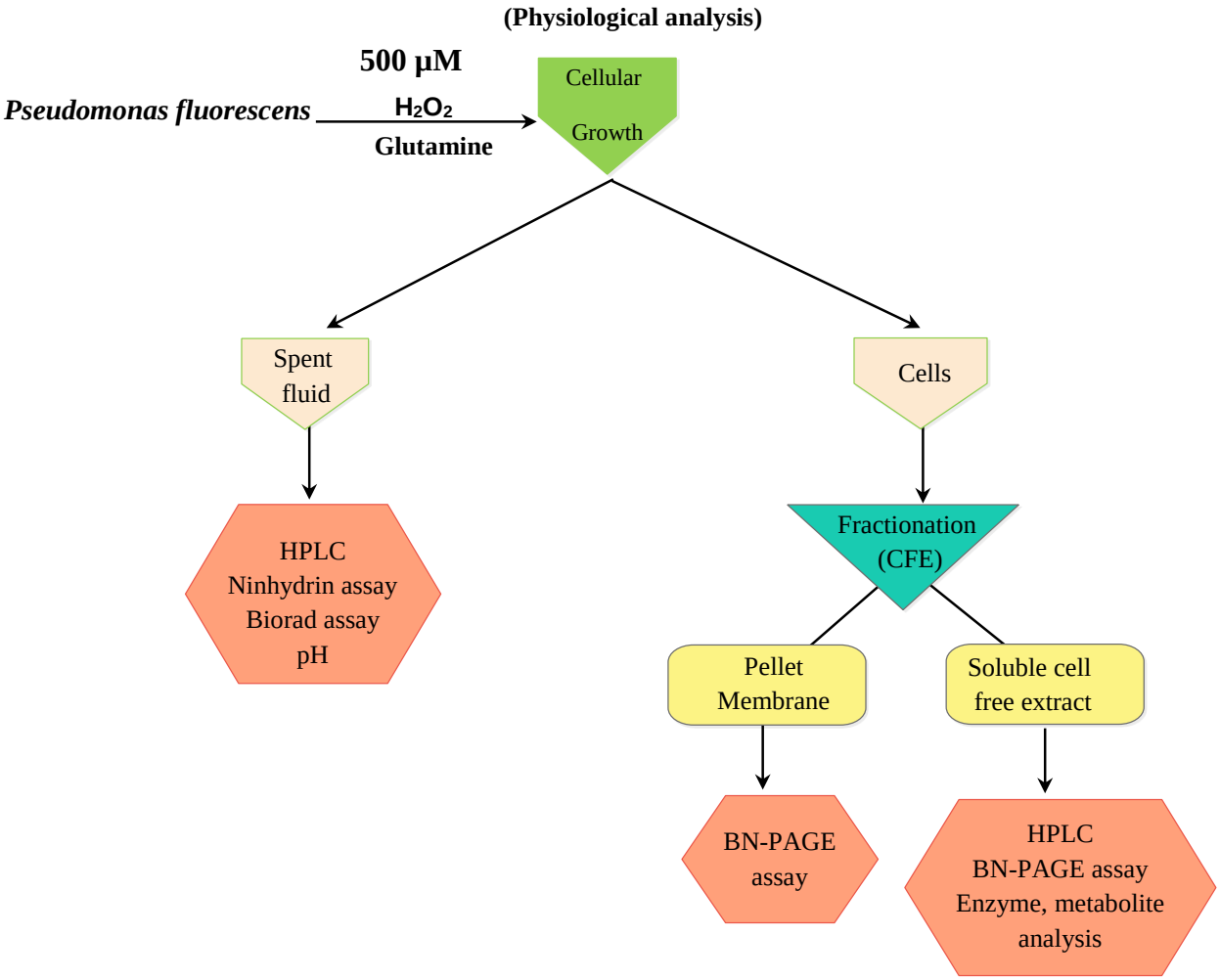

Figure 1.1-12: A brief experimental design of the project.

CHAPTER 2: The role of glutamine synthetase in energy production and glutamine metabolism during oxidative stress

The role of glutamine synthetase in energy production and glutamine metabolism during oxidative stress.

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2.1. Abstract:

Oxidative stress is known to severely impede aerobic ATP synthesis. However, the metabolically-versatile *Pseudomonas fluorescens* survives this challenge by invoking alternative ATP-generating networks. When grown in a medium with glutamine as the sole organic nutrient in the presence of H_2O_2, the microbe utilizes glutamine synthetase (GS) to modulate its energy budget. The activity of this enzyme that mediates the release of ATP trapped in glutamine was sharply increased in the stressed cells compared to the controls. The enhanced activities of such enzymes as acetate kinase (ACK), adenylate kinase (AK) and nucleoside diphosphate kinase (NDPK) ensured the efficacy of this ATP producing-machine by transferring the high energy phosphate. The elevated amounts of phosphoenol pyruvate carboxylase (PEPC) and pyruvate orthophosphate dikinase (PPDK) recorded in the H_2O_2 exposed cells provided another route to ATP independent of the reduction of O_2. This is the first demonstration of a metabolic pathway involving GS dedicated to ATP synthesis. The phospho-transfer network that is pivotal to the survival of the microorganism under oxidative stress may reveal therapeutic targets against infectious microbes reliant on glutamine for their proliferation.

Keywords: Glutamine synthetase, ATP budget, oxidative stress, metabolic networks.

2.2. Introduction:

Adenosine triphosphate (ATP) is the universal chemical energy that is utilized in all biological systems. This phosphate-rich nucleotide is usually generated by the addition of phosphate to adenosine diphosphate (ADP) during oxidative phosphorylation, an O_2-dependent process. ATP can also be produced by a process referred to as substrate-level phosphorylation (Appanna et al. 2016; Auger and Appanna 2015; Coustou et al. 2003). In this instance, a moiety with a high-energy phosphate donates its phosphate to ADP, a metabolic event that does not necessitate the participation of O_2. Organisms facing oxidative challenge do not only have to detoxify the reactive oxygen species (ROS) but have to ensure a constant supply of ATP since the classical route to synthesizing this moiety becomes an easy victim of oxidative stress (Zeller and Klug 2004; Salin et al. 2015; Bailey-Serres et al. 2012). It is well-documented that the tricarboxylic acid (TCA) cycle, a supplier of reducing factors is severely impeded and the electron transport chain (ETC) that propels ATP production is ineffective (Bignucolo et al. 2013; Singh et al. 2007).

In such circumstances where ROS levels are elevated or oxygen tension is low, living organisms resort to glycolysis, a metabolic network dedicated to the production of the high-energy biomolecule known as phosphoenol pyruvate (PEP). This moiety can be readily converted into ATP and pyruvate with the aid of the enzyme pyruvate kinase (PK) and the participation of ADP. The nutritionally-versatile *Pseudomonas fluorescens* and numerous other cellular systems have been shown to adopt this strategy when confronted with oxidative stress (Auger and Appanna 2015; Appanna et al. 2016; Baily et al. 2011; Heiden et al. 2010). Following the generation of PEP, it is transformed into ATP, a process that is mediated by the enzymes pyruvate orthophosphate dikinase (PPDK) and phosphoenol pyruvate synthase (PEPS). The latter utilizes Pi while the former invokes the use of PPi as the co-factor. This stratagem renders this energy-making machine

efficient and ensures the survival of these organisms despite the ineffectiveness of oxidative phosphorylation (Dzeja and Terzic 2003; Auger et al. 2011; Chastain et al. 2011). For instance, *Trypanosoma brucei* that triggers the sleeping sickness disease invokes the participation of an ATP-forming engine orchestrated by PPDK (Coustou et al. 2003).

As part of our effort to decipher how organisms proliferate in an environment where they are bombarded by ROS or where the oxygen level is low, we have evaluated the molecular response of *P. fluorescens* exposed to toxic amounts of H_2O_2. Glutamine, a crucial amino-acid in all organisms was utilized as the sole organic nutrient. Besides providing the carbon backbone to fuel energy production, this moiety is critical in the biosynthesis of key biomolecules such as nucleic acids, polyamines, asparagine and amino-sugars (Robert 1990; Altman el at. 2016; Hensley et al. 2013). Most cancer cells are uniquely programmed to consume glutamine in order to support their rapid proliferation using essentially glutaminase (GLS) in the degradation of this moiety (DeBerardinis and Cheng 2010; Cantor and Sabatini 2012; Zhao et al. 2011). However, in this study we show that H_2O_2-challenged *P. fluorescens* fulfills its energy need by liberating the ATP stored in glutamine via glutamine synthetase (GS). The activity of this enzyme was up-regulated during oxidative stress. Furthermore, the significance of numerous phospho-transfer networks that ensure a constant supply of ATP and the importance of energy production by substrate-level phosphorylation are also discussed.

2.3. Material and methods:

2.3.1.Bacterial culturing conditions:

The bacterial strain *Pseudomonas fluorescens* (ATCC 13525) was obtained from the American type culture collection. It was grown in defined mineral media, consisting of Na_2HPO_4 (6 g), KH_2PO_4 (3 g), and $MgSO_4.7H_2O$ (0.2 g), at a pH of 6.8. Glutamine (2.7gm) was used as the sole source of carbon and nitrogen. The trace elements were added as described in Mailloux et al. (2008). Media were dispensed into 200 mL aliquots in two 500 mL Erlenmeyer flasks and autoclaved for 20 min at 121 °C. The cultures were inoculated with 1 mL of bacteria (450 µg protein equivalent) grown to stationary phase in a control medium. Hydrogen peroxide (500 µM) was introduced in the stressed culture as described in (Alhasawi et al. 2015). Cultures were aerated in a gyratory water bath shaker, model 76 (New Brunswick Scientific) at 26°C at 140 rpm. The cells and spent fluid were isolated at the stationary phase of growth (24h for control and 28h growth for the H_2O_2 stressed cultures) for metabolomic and enzymatic analyses Following the harvesting of cells by centrifugation, they were treated with 0.5 N NaOH. Cellular biomass was monitored by measuring solubilized protein using the Bradford assay (Bradford 1976).

2.3.2.Cell fractionation and regulation experiments

The cells in the control and stressed cultures were pelleted by centrifugation at 10,000 x g for 20 min at 4 °C. Cells were washed with 0.85 % NaCl and re-suspended in a cell storage buffer (CSB) consisting of 50 mM Tris-HCl, 5 mM $MgCl_2$ and 1 mM phenylmethylsulphonyl fluoride (PMSF). The cells were disrupted by sonication for 15 s, 3 times with 3 min wait periods. The cell free extract (CSB), and membrane fraction were obtained by centrifugation for 3 h at 180,000 x g

at 4 °C. Unbroken cells were removed via centrifugation at 10,000 x g. The membrane fraction was resuspended in 1 mL of (CSB). The Bradford assay was utilized to determine the protein concentration with serum bovine albumin as the standard.

To verify the adaptive and reversible metabolic changes triggered by the oxidative stress, the control cells were incubated for 8 h in the H_2O_2 media whereas the H_2O_2-stressed cells were incubated for 8 h in control media as described in (Alhassawi et al. 2014). The cellular fractions were subsequently analyzed for metabolites and enzymatic activities.

2.3.3.Metabolite analysis:

Metabolite levels were recorded using high performance liquid chromatography (HPLC). Cells cultured in control and H_2O_2-stressed conditions, were harvested at similar growth phase and lysed by sonication. The soluble cellular fractions were immediately analyzed following treatment with methanol or boiling for 10 min. The samples were injected into Alliance HPLC with C18 reverse-phase column (Synergi Hydro-RP; 4μm; 250× 4.6 mm ,Phenomenex) attached to a Waters dual absorbance detector. A mobile phase consisting of 20 mM K_2HPO_4 (pH 2.9) was used at a flow-rate of 0.7 mL/min at ambient temperature to separate the substrate and products, which were measured at 210 nm for carboxylic acids and 280 nm for nucleotides. Metabolites were identified using known standards and spiking samples with known metabolite of interest. Peaks were quantified using the Empower software (Waters Corporation). Select activity bands were also excised and incubated with the appropriate substrates. The corresponding products were monitored by HPLC. Glutamine synthetase (GS) bands were cut from the control and stress and incubated in 1 mL mixture of 2 mM glutamine, 0.5 mM ADP and 0.5 mM Pi. After 30 min of incubation, 100 μL of the sample was removed and diluted with Milli-Q water for HPLC analysis.

2.3.4.Functional proteomic studies

Blue native polyacrylamide gel electrophoresis (BN-Page) was performed as described in (Auger and Appanna 2015; Mailloux et al. 2008; Schagger and Von Jagow 1991). For these assays, a 4-16% gradient gel was prepared and protein (4 g/L) was prepared in blue native buffer (400 mM 6-amino hexanoic acid, 50 mM Bis-Tris, pH 7.0). To solubilize membrane bound proteins and in order to ensure optimal protein separation, a final concentration of 1% dodecyl-maltoside was added to membrane fractions. Protein samples (60µg) were loaded into each well and electrophoresed at 4 °C under native conditions. Following electrophoresis, gels were incubated in reaction buffer (25 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4) and in-gel visualization of enzyme activity was determined by linking the formation of NAD(P)H to 0.2 mg/mL of phenazine methosufate (PMS) and 0.4 mg/mL of iodonitrotetrazolium (INT), or coupling the formation of $NAD(P)^+$ to 16.7 g/mL, 2,6-dichloroindophenol (DCPIP) and 0.4 mg/mL INT. Glutamine synthetase (GS) was visualized using a reaction mixture consisting of 5 mM glutamine, 0.5 mM sodium phosphate, 0.5 mM nicotinamide adenine dinucleotide (NAD), 1 mM ADP, 10 units of glutamate dehydrogenase (GDH), 0.2 mg/mL PMS and 0.4 g/mL INT. Glutaminase (GLS) was detected utilizing a reaction mixture consisting of 5 mM glutamine, 0.5 mM NAD, 10 units of GDH, 0.2 mg/mL PMS and 0.4 g/mL INT. In both these instances, the formation of glutamate was profiled. The formation of ATP was visualized in the case of GS with hexokinase as in (Singh et al 2009). Complex I was detected by the addition of 5 mM KCN, 5 mM NADH, and 0.4 g/mL INT in the reaction buffer. The activity of NADP-dependent isocitrate dehydrogenase (ICDH-NADP) was visualized using a reaction mixture consisting of 5 mM isocitrate, 0.5 mM NADP, 0.2 mg/mL PMS and 0.4 mg/mL INT. The same reaction mixture was utilized for ICDH-NAD except 0.5 mM NAD was utilized in lieu of NADP. The reverse reaction of this enzyme was monitored by

incubating the gel in 5 mM αketoglutarate, 10 mM $NaHCO_3$ and 0.5 M NADH in the presence of DCPIP and INT. αketoglutarate dehydrogenase (αKGDH) was visualized by using reaction contained 5 mM α ketoglutarate, 0.5 mM NAD, 1 mM CoA, 0.2 mg/mL PMS and 0.4 mg/mL INT. Pyruvate phosphate dikinase (PPDK) was monitored using reaction mixture consisting of 5 mM phosphoenol pyruvate (PEP), 0.5 mM AMP, 0.5 mM sodium pyrophosphate (PPi), 0.5 mM NADH, 10 units of lactate dehydrogenase (LDH), 0.0167 mg/mL of DCPIP and 0.4 mg/mL of INT. Phosphoenolpyruvate carboxylase (PEPC) was assayed by the addition of 0.5 mM PEP, 5 mM HCO_3, 5 units of malate dehydrogenase (MDH), 0.4 mg/mL INT and 0.0167 mg/mL of DCPIP. Phosphoenolpyruvate synthase (PEPS) was imaged using the reaction mixture containing of 5 mM PEP, 0.5 mM inorganic phosphate (Pi), 0.5 mM NADH, 10 units LDH, 0.0167 of DCPIP and 0.4 mg/mL of INT. The activity of adenylate kinase (AK) was probed with a reaction mixture containing 5 mM ADP, 5 mM glucose, 0.5 mM NADP, 5 units of hexokinase, 5 units of G6PDH, 0.4 mg/mL INT and 0.2 mg/mL PMS. The activity of nucleoside diphosphate kinase (NDPK) was monitored using reaction mixture consisting of 0.5 mM ADP, 0.5 mM $NADP^+$,10 mM glucose, 5 units of G6PDH, 5 units of hexokinase, 5 mM of GTP, 0.4 mg/mL INT and 0.2 mg/mL PMS. Acetate kinase (ACK) was analyzed in gel with a reaction mixture consisting of 5 mM acetyl phosphate, 1 mM ADP, 5 mM glucose, 10 units of G6PDH, 10 units of hexokinase, 1 mM NADP, 0.2 mg/mL PMS and 0.4 mg/mL of INT. Destaining solution (40% methanol and 10% glacial acetic acid) was used to stop the reactions where appropriate. Coomassie staining was used to ensure equal protein loading. As pyruvate carboxylase (PC) activity did not change significantly, this enzyme was also utilized as a loading control (Alhasawi et al. 2014). Densitometry was performed using image J for windows in order to obtain comparative values of enzymatic activity in control and stressed cells.

The specificity of enzymatic reaction was further confirmed by performing in-gel reactions in the absence of a substrate or by the addition of the inhibitors (L-alanine for glutamine synthetase or rotenone for Complex I). Spectrophotometric data for ICDH-NAD was obtained by incubation 0.2 mg (protein equivalent) of membrane fraction from control and H_2O_2-traeted cells with 2 mM isocitrate and 0.5 mM NAD for 1 min and monitoring NADH formation. A similar reaction was used for GDH, but isocitrate was replaced with glutamate. KGDH activity was performed as described in (Bignucolo et al. 2013). NADH production were monitored at 340 nm over the course of a minute. For pyruvate carboxylase (PC) analysis, the membrane CFE was given 2 mM pyruvate, 0.5 mM ATP, 0.5 HCO_3, 10 units of MDH and 0.5 mM NADH. Negative controls were performed without the substrate or cofactors. The GS activity was measured using γ-glutamyl-hydroxamate assay. The reaction measured the conversion of glutamate to γ-glutamyl-hydroxamate. It was visualized using 0.5 mL of reaction mixture consisting of 300 mM glutamate, 55 mM $MgCl_2$, 46 mM hydroxylamine-HCl, and 92 mM imidazole, pH 7.0. The reaction was initiated by addition of ATP and substituting water for ATP in blanks. The reaction was incubated for 5 min, then was stopped with 1 mL of mixture consist of 55 g $FeCl_3$, 20 g trichloroacetic acid, and 21 mL of 12%HCl. The absorbance of the complex of γ-glutamyl- hydroxamate-iron with extinction coefficient 7.7×10^5 was measured at 540 nm (Eid et al. 2004; Robinson et al. 2001). The specific activity of GS measured μmole of γ-glutamyl-hydroxamate $min^{-1}mg^{-1}$.

2.3.5.Statistical analysis:

Data were expressed as means ± standard deviations. Percent change was calculated where appropriate in order to account for individual variation and to provide a better measure of change

in activity. Data were checked for significance using the student *t* test ($p \leq 0.05$). All experiments were performed in at least biological duplicate and repeated thrice.

2.4. Results:

When subjected to an oxidative environment with glutamine as the sole carbon and nitrogen source, *Pseudomonas fluorescens*, experienced a slower growth rate compared to the control cells. However, at stationary phase of growth the cellular biomass in the control and H_2O_2-challenged media was relatively similar (Fig. 1A). Analysis of select metabolites at the stationary growth phase revealed marked variations in the cellular extracts. While pyruvate levels were at least 2-fold higher in the control CFE compared to the stressed cultures, the latter was characterized by higher amounts of αKG, succinate and glutamate (Fig. 1B). There were also significant changes in adenosine nucleotides in the CFE of the H_2O_2-treated cultures. ATP and AMP levels were more abundant in stressed cultures (Fig. 1C).

As one of our objective was to evaluate the metabolic pathways involved in the production of energy under oxidative stress, enzymes participating in tricarboxylic acid cycle (TCA) and oxidative phosphorylation, two networks most aerobic organisms utilize to generate ATP were probed. Complex I, a prominent indicator of oxidative phosphorylation was very active in the control membrane fraction but was barely evident in the stressed conditions (Fig. 2A). While the TCA cycle enzymes like αKGDH was sharply diminished in stressed cultures, there was a notable increase in ICDH-NAD compared to the control (Fig. 2B, and Table 1). The latter is known to be prominent in the reverse direction during glutamine metabolism under limited oxygen conditions (Filipp et al. 2012; Michalak et al. 2015). Indeed the bands corresponding to the reverse reaction where αKG, HCO_3 and NADH were included in the reaction mixture were similar to the forward reaction (Fig. 2B). As expected, ICDH-NADP was elevated in the stressed cultures (Fig. 2C).

NADPH is an important anti-oxidant. However, in this instance the reverse reaction was barely evident (data not shown). Hence, it became clear that the stressed cells were invoking a metabolic pathway other than oxidative phosphorylation to fulfill their need in ATP. Since pyruvate carboxylase (PC) did not change significantly in the control and stressed cells, it was utilized as a loading control (Fig. 2D)

As glutamine was the only source of organic nutrient, it became critical to analyze how this amino acid was being metabolized. Glutaminase (GLS) and glutamine synthetase (GS) are two key enzymes that can contribute to the metabolism of glutamine (Labow et al. 2001). These enzymes were elevated in the stressed cells (Fig. 3A). When the control cells and the stressed cells obtained at stationary were analyzed for GLS, the stressed-soluble CFE revealed a more intense band than the control, an observation that was not surprising (Fig. 3A). However, when probed for GS, an enzyme that necessitates the participation of ADP in the degradation of glutamine into glutamate, ATP and NH_3, this enzyme was more prominent in the stressed cells (Fig. 3B). The ability of the enzyme to produce both glutamate and ATP was revealed by formazan precipitation. Glutamate was detected with the aid of glutamate dehydrogenase (GDH) while the presence of ATP at the site GS activity in the gel was visualized by including hexokinase and glucose 6-phosphate dehydrogenase in the incubation mixture. Cell free extract isolated from the H_2O_2-grown cells transferred in the control medium was characterized with a marked reduction in both GLS and GS activity (Fig. 3A, B). The reverse was true when the CFE obtained from the control cells incubated in the H_2O_2 medium (Fig. 3A, B). The activity band attributable to GS also increased with incubation time and revealed an optimal activity at 28 h of growth (Fig. 3C). This activity was arrested in the presence of such inhibitors as alanine (Fig. 3B).

The activity of GS was also monitored by its ability to produce γ-glutamyl hydroxamate, a product that is known to give a characteristic colour upon complexing with iron. A more than 2-fold increase activity was recorded in the cultures subjected to H_2O_2 compared to the controls (Fig. 3D). Incubation of the activity band with glutamine and ADP yielded ATP and glutamate as revealed by HPLC (Fig. 3E). This finding clearly pointed to the relationship between elevated GS activity and oxidative stress. Glutamate dehydrogenase, GDH an enzyme that deaminates glutamate with concomitant reduction of either NADP or NAD was markedly upregulated in activity in the stressed cells (Fig. 3F, and Table 1). Thus, it appears that the H_2O_2-challenged cells were utilizing glutamine to replenish its ATP budget.

Since ADP was an important ingredient fuelling the degradation of glutamine, it was important to assess how this nucleotide was being generated. Acetate kinase (ACK), is an enzyme that mediates the phosphorylation of acetate coupled to the formation of ADP. This enzyme was found be activated in the stressed cells. Likewise, other enzymes that can contribute to the ATP and ADP homeostasis were also positively affected by the oxidative environment. Adenylate kinase (AK) and nucleoside diphosphate kinase (NDPK) activities were marked enhanced in the membrane CFE obtained from the cells cultured in H_2O_2 media compared to the controls (Fig. 4A).

The energy budget was also supplemented with the substrate level phosphorylation strategy anchored by PEP. The production of this high energy phosphate was sharply increased in the stressed cells as were activities of PEPS, PPDK, and PK. These enzymes readily phosphorylate AMP and ADP into ATP. Indeed, PEPS and PPDK activities were higher in the stressed cells (Fig. 4B). A 4-fold increase in the case of the latter enzyme was observed (Fig. 4B).

2.5. Discussion:

The results presented in this report point to the ability of *P. fluorescens* to survive an oxidative environment by catabolising glutamine in such a manner as to maximize ATP production in order to counter an ineffective electron transport chain (ETC) promoted by H_2O_2. This process is propelled by glutamine synthetase (GS), an enzyme that effectively releases the ATP trapped in glutamine, unlike glutaminase (GLS) known for its ability to only liberate glutamate and NH_3. The up-regulation of GLS has been widely reported in numerous cells subjected to oxidative stress. It provides α ketoglutarate (KG), a moiety that is subsequently utilized in a variety of catabolic and anabolic processes including those responsible for the cellular energy budget (Amelio et al. 2014; Cooper and Jeitner 2016). Glutamine synthetase (GS) was characterized by an increased in activity under oxidative stress. This enzyme is known to play a crucial role in nitrogen homeostasis and in most organisms it is invoked to convert glutamate into glutamine with the aid of ATP and NH_3. The glutamine is subsequently utilized in the synthesis of nucleic acids, NAD and other essential metabolites necessary for cellular proliferation (Cairns et al. 2011; Lane and Fan 2015; Boza et al. 2000; De Ingeniis et al. 2012).

However, in this study GS may be having an entirely disparate function as there was an abundance of glutamine. In fact, this amino acid was the sole source of carbon and nitrogen. Hence, the primary goal of this enzyme was not to synthesize glutamine but to utilize it. The degradation of glutamine promoted by GS not only supplies glutamate but also releases ATP, a pivotal ingredient during oxidative stress. The production of ATP is severely hampered as the numerous enzymes of the TCA cycle and the electron transport chain (ETC) are susceptible to ROS (Auger and Appanna 2015; Shimizu 2013; Mailloux et al. 2007). This strategy may be advantageous as it helps unlock the energy sequestered in the amide bond of glutamine. Although this enzyme has

been traditionally assigned the task of converting glutamate into glutamine, the data in this report would argue for a role other than the synthesis of the amino acid since it was abundantly present in the medium. This attribute of GS in supplementing the ATP budget of an organism under H_2O_2 stress has hitherto not been reported (Rana et al. 2008). The benefits imparted by the up-regulation of this ATP-producing enzyme during a situation where energy formation via oxidative phosphorylation is sharply compromised may provide an attractive stratagem in environments where oxygen is limiting and glutamine is plentiful. Glutaminase (GLS) that has been widely reported in the literature to provide an easy access to glutamate during anaerobic conditions is devoid of the ATP-generating attribute of GS. This nucleotide coupled to the formation of glutamate may be central to the survival of *Pseudomonas fluorescens* subjected to H_2O_2 in a glutamine-rich environment. Glutamate can then be processed by glutamate dehydrogenase (GDH), an enzyme that was significantly higher in the stressed cultures. Glutamate, a product that was generated by both GLS and GS was readily converted into KG by GDH. Indeed, the levels of glutamate, KG and succinate were higher in the cell free extracts obtained from the stressed cells compared to the control. The reduction of oxidative phosphorylation as evidenced by an ineffective Complex I coupled with the diminished activity of αKGDH, resulted in the transformation of αKG into isocitrate. This was mediated by ICDH-NAD, an enzyme that was markedly increased in the H_2O_2-challenged cells, a metabolic adaptation not uncommon during oxidative stress and anaerobiosis. In fact numerous organisms including cancer cells are known to resort to a reverse TCA cycle into to satisfy their need for essential metabolites in order to survive (Michalak et al. 2015; Filipp et al., 2012). Hence, it is quite likely that the KG liberated following the catabolism of glutamine is converted into isocitrate in *P. fluorescens* subjected to an oxidative challenge. The increased activity of ICDH-NADP, an isocitrate-utilizing enzyme would corroborate such a

postulation. The NADPH and KG generated would act as potent antioxidants, ingredient crucial for survival during a H_2O_2 stress (Lemire et al. 2010; Mailloux et al. 2009).

In order for GS to act as a potent generator of ATP in an environment with copious amounts of glutamine, it is important that ADP a key nucleotide fuelling this reaction needs to be adequately supplied. The increased activity of enzymes like AK, NDPK, and ACK may contribute to this effort. Indeed their ability to phosphorylate various substrates with the participation of ATP and the concomitant formation of ADP would provide an effective metabolic route to accomplish this task. These phospho-transfer networks orchestrated by the GS-mediated catabolism of glutamine can help compensate for the ineffective oxidative phosphorylation observed during oxidative stress and diminished oxygen tension. The role of phospho-networks and substrate-level phosphorylation in enabling the survival of organisms compromised in their ability of acquiring ATP by oxidative phosphorylation have been reported in numerous organisms (Auger and Appanna 2015; Appanna et al. 2016; Coustou et al. 2003). In this instance, PEP appears to be a critical conduit for these processes. In the present report, the ability of *P. fluorescens* to activate the synthesis of PEP via the enzyme PEPC may be an important contributor to this stratagem. The fixation of PEP into ATP, a process mediated by the up-regulation of activities of the ADP and AMP-dependant kinases affords an elegant route to replenish the ATP budget in this H_2O_2-challenged situation. Hence, this H_2O_2 –triggered metabolic shift anchored by GS is an intriguing ATP-generating machine designed to offset the ineffective oxidative phosphorylation.

In conclusion, the up-regulation of GS provides an effective means of combatting the ATP deficit in this microbe assaulted by ROS. The extraction of ATP from this abundant nutrient coupled to the phospho-relay networks that modulate the levels of AMP and ADP reveal an intricate scheme this organism invokes to fend off oxidative challenge (Figure 5). These metabolic

modules may provide interesting targets to quell infectious parasitic organisms seeking O_2-independent routes to proliferate in their host, as glutamine is the most abundant amino acid in humans.

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2.7. Figures, legends and table

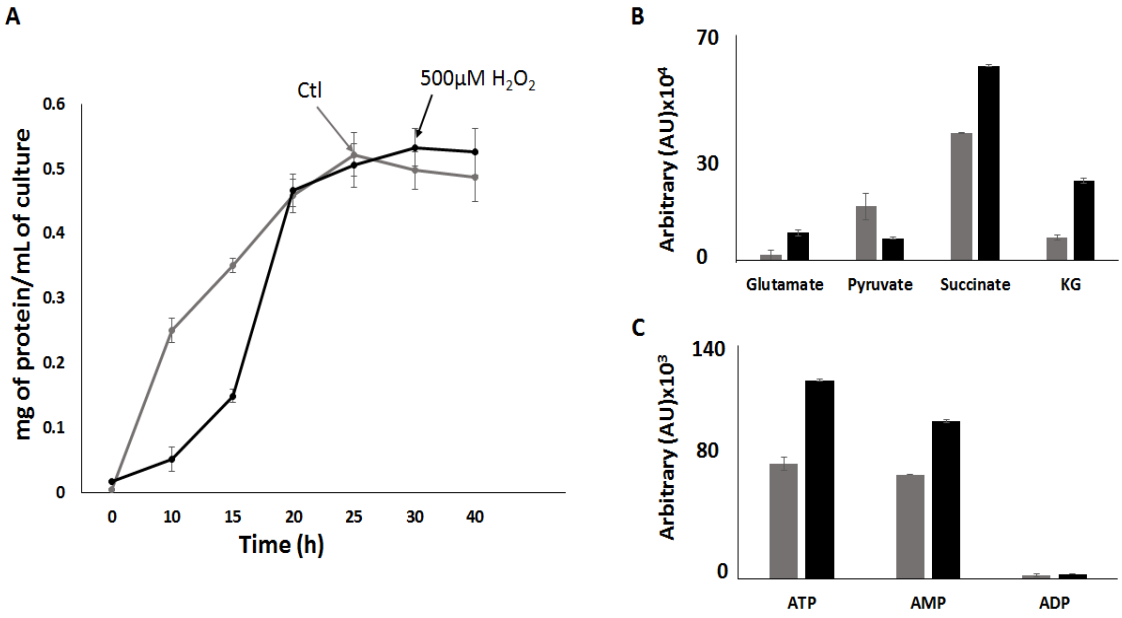

Figure 2.7-1: Bacterial biomass and functional metabolomics in (•) control (•) 500 µM H2O2-stressed cultures. A : Microbial growth. B : Metabolite profile in soluble cell free extract. C : Adenosine nucleotide levels reflective of cellular energy. (n=3).

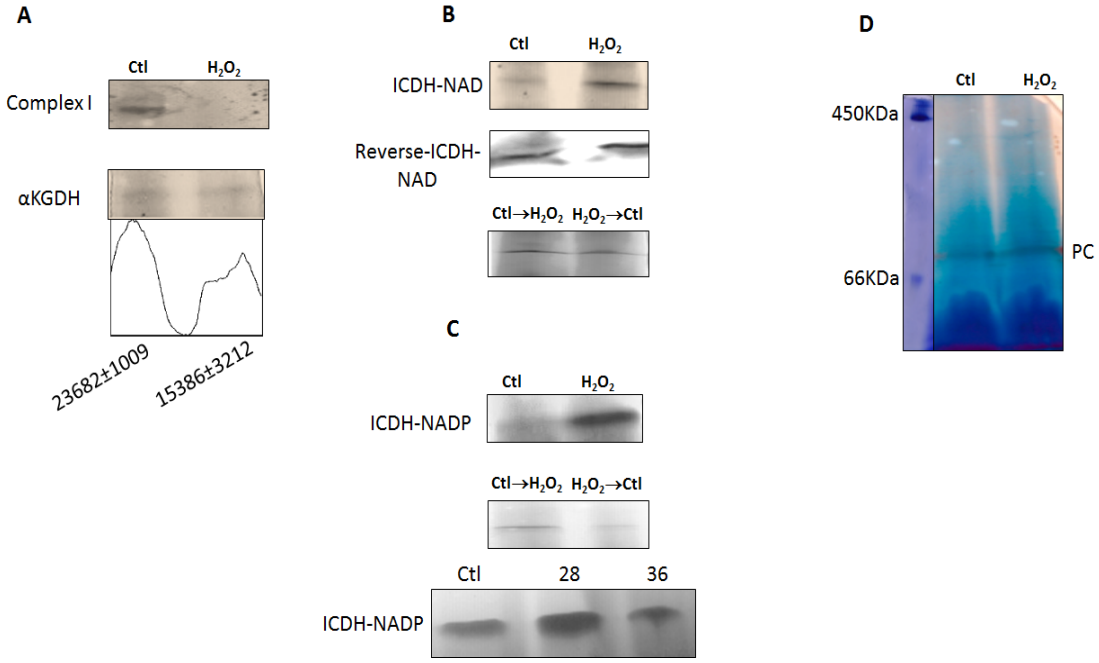

Figure 2.7-2 In gel activity analyses of metabolic enzymes. A: Complex I, αKetoglutarate dehydrogenase (αKGDH) . B : Isocitrate dehydrogenase (ICDH-NAD. (F/R) D : ICDH-NADP. E: Pyruvate carboxylase (PC) loading control. (Gels are representative of 3 independent experiments. Ctl = Control; H_2O_2-treated). F= Forward reaction; R= Reverse reaction. Densitometry was performed using Image J for windows. *Denotes a statistically significant differences compared with the control ($P \leq 0.05$).

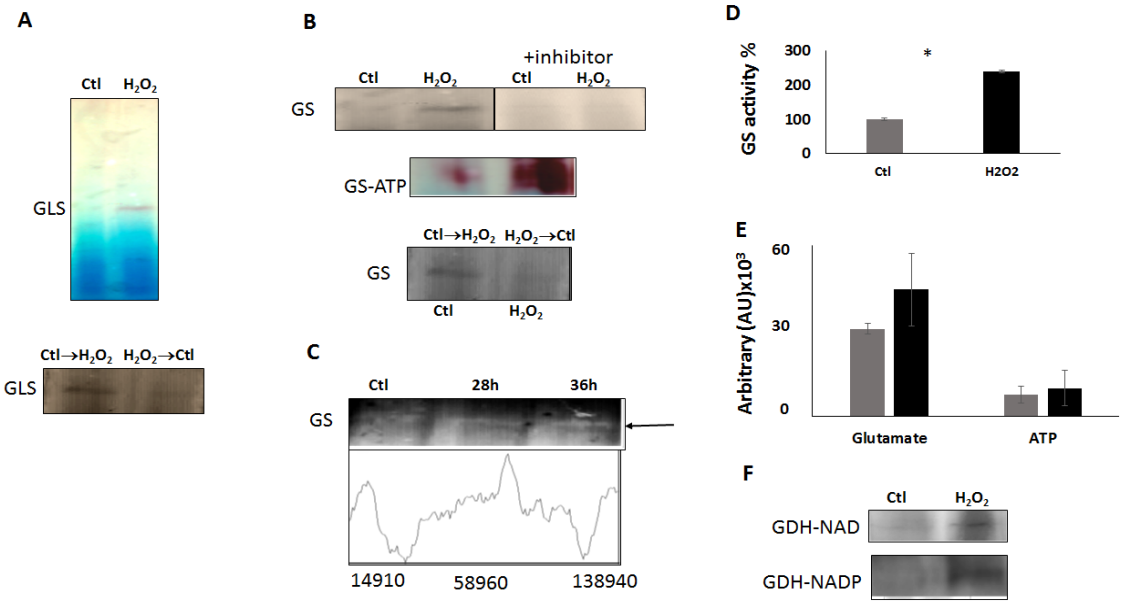

Figure 2.7-3: Glutamine metabolism in control and H_2O_2-stressed *P. fluorescens*. A: In-gel enzymatic activity of glutaminase (GLS) and in-gel activity of GS when control cells were incubated in H_2O_2-cultures and H_2O_2-treated cells were exposed to control media. B: In-gel enzymatic activity of glutamine synthetase (GS) with and without the presence of inhibitor (alanine). C: Time profile of GS activity in stressed cells. D: γ-glutamyl hydroxatmate assay for GS. E: HPLC analysis of excised band (GS) incubated in 2 mM glutamine, 0.5 mM ADP and 0.5 mM Pi for 30 min. Both glutamate and ATP formation were detected. F: In-gel glutamate dehydrogenase (GDH) (NAD)P activity. (Gels are representative of 3 independent experiments. Ctl = Control; H_2O_2-treated). 100% glutamine synthetase (GS) activity corresponds to (151.9 μmole γ-glutamyl hydroxatmate produced $min^{-1}mg^{-1} \pm 2\%$). n=3. *Denotes statistically significant differences compared with the control ($P \leq 0.05$). (•) control (•) 500 μM H_2O_2-*P. fluorescens*; the variation in the error bar may be associated with the imprecision in excising the activity bands .

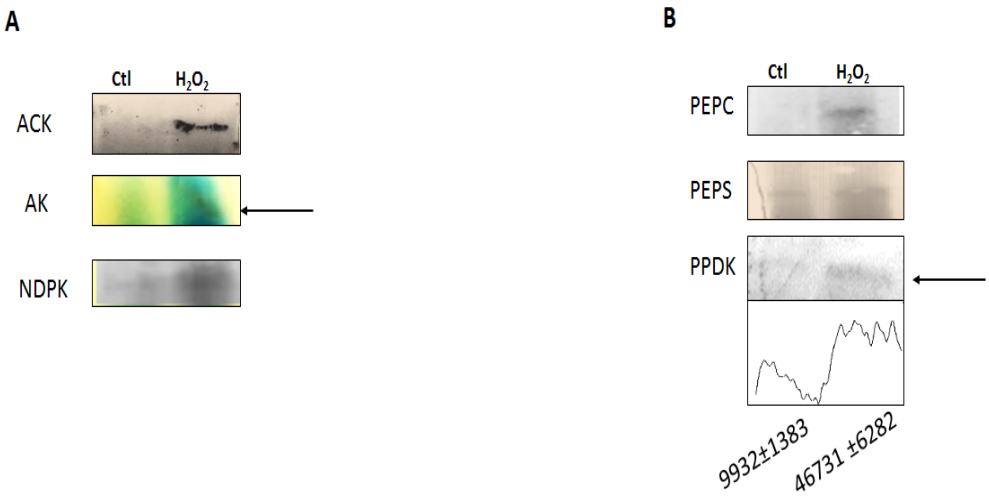

Figure 2.7-4: Enzymes involved in phosphotransfer networks in (•) control (•) 500 µM H_2O_2-*P. fluorescens*. A: Adenylate kinase (AK), nucleoside diphosphare kinase (NDPK) and acetate kinase (ACK). B: Phosphoenolpyruvate carboxylase (PEPC), pyruvate orthophosphate dikinase (PPDK) and Phosphoenolpyruvate synthase (PEPS). (Gels are representative of 3 independent experiments. Ctl = Control; H_2O_2-treated). Densitometry was performed using Image J for windows.

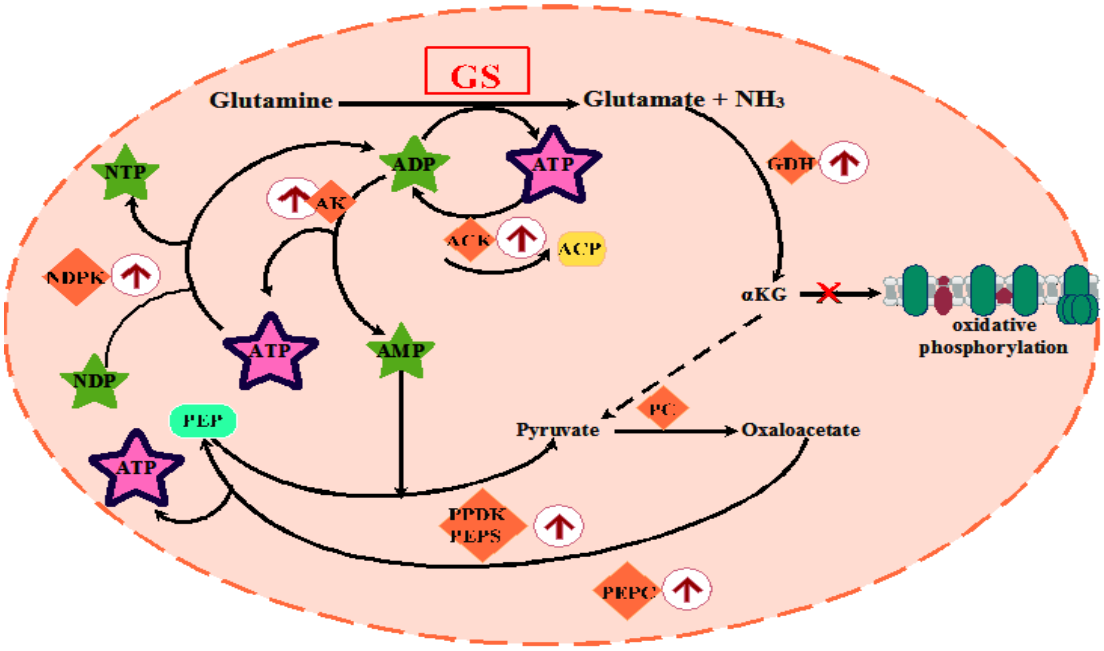

Figure 2.7-5: Schematic depiction of the role of GS in ATP homeostasis in *P. fluorescens* during oxidative stress. Glutamine synthetase (GS); adenosine diphosphate (ADP); adenosine triphosphate (ATP); glutamate dehydrogenase (GDH); alpha-ketoglutarate (αKG); acetate kinase (ACK); acetyl phosphate (ACP); adenosine monophosphate (AMP); adenylate kinase (AK); nucleoside triphosphate (NTP); nucleoside diphosphate kinase (NDPK); nucleoside diphosphate (NDP); pyruvate carboxylase (PC); pyruvate orthophosphate dikinase (PPDK); phosphoenolpyruvate synthase (PEPS); phosphoenol pyruvate (PEP). ↑=Increase; ✕=decrease.

Table 2.7-1: Enzymatic activities in CFE from control and H_2O_2-*P. fluorescens* at the same growth phase (24 h and 28 h respectively) as monitored by spectrometry**.**

ENZYMES	CTL	H_2O_2
NAD- isocitrate (ICDH)[a]	0.63±0.12	1.03±0.2*
NAD-glutamate dehydrogenase (GDH)[a]	0.731± 0.08	1.12± 0.018
α ketoglutarate dehydrogenase (KGDH)[a]	0.62±0.15	0.345±0.02*
Pyruvate carboxylase (PC)[b]	2.6±0.37	2.65±0.06

[a] µmol NADH produced min^{-1} mg $protein^{-1}$ as monitored at 340 nm (n = 3 ± standard deviation).
[b] µmol NADH consumed min^{-1} mg $protein^{-1}$ as monitored at 340 nm (n = 3 ± standard deviation).
*Denotes a statistically significant differences compared with the control ($P \leq 0.05$).

CHAPTER 3: Conclusions, future research and general bibliography

3.1. Conclusions

Although ROS detoxification strategies are critical for survival in oxidative environment, it is very important to evaluate other processes that are responsible to combat this situation. ATP is a crucial ingredient and its production is sharply affected during oxidative stress. Enzymes that mediate ATP production via the TCA cycle and the electron transport chain (ETC) are ineffective. In this instance, Aconitase (ACN), Complex I, Complex II (succinate dehydrogenase), Complex III, and Complex IV all depend of Fe as iron-sulfur cluster in order to perform their biological functions. The exposure of the cell to a high oxidative stress, disrupts the iron-sulfur cluster and impairs the activity of these proteins. The ROS displace the Fe in the iron-sulfur cluster and impede oxidative phosphorylation. Thus, ATP production is arrested. In this study, the ATP budget was supplemented by substrate level phosphorylation. This network was driven by glutamine synthetase (GS). The microorganism under ROS stress has to seek alternative ATP-generating pathways. In this instance it utilized GS to release the stored energy that is trapped in the amide bond of glutamine. Thereby, GS generates an effective supply of ATP in an environment where glutamine is plentiful. Once this strategy was adopted the microorganism required constant supply of ADP to generate ATP. The up-regulated phospho-transfer network involving acetate kinase (ACK), adenylate kinase (AK) and nucleotide diphosphate kinase (NDPK) become a potent source of ADP, as the ATP generated by GS is fixed. GS is a vital enzyme dedicated to extract maximum ATP with aid of phospho-transfer networks to fend off oxidative stress (Figure 3.1-1). This study is the first demonstration of the role of GS in contributing to the ATP budget of an organism challenged by oxidative stress.

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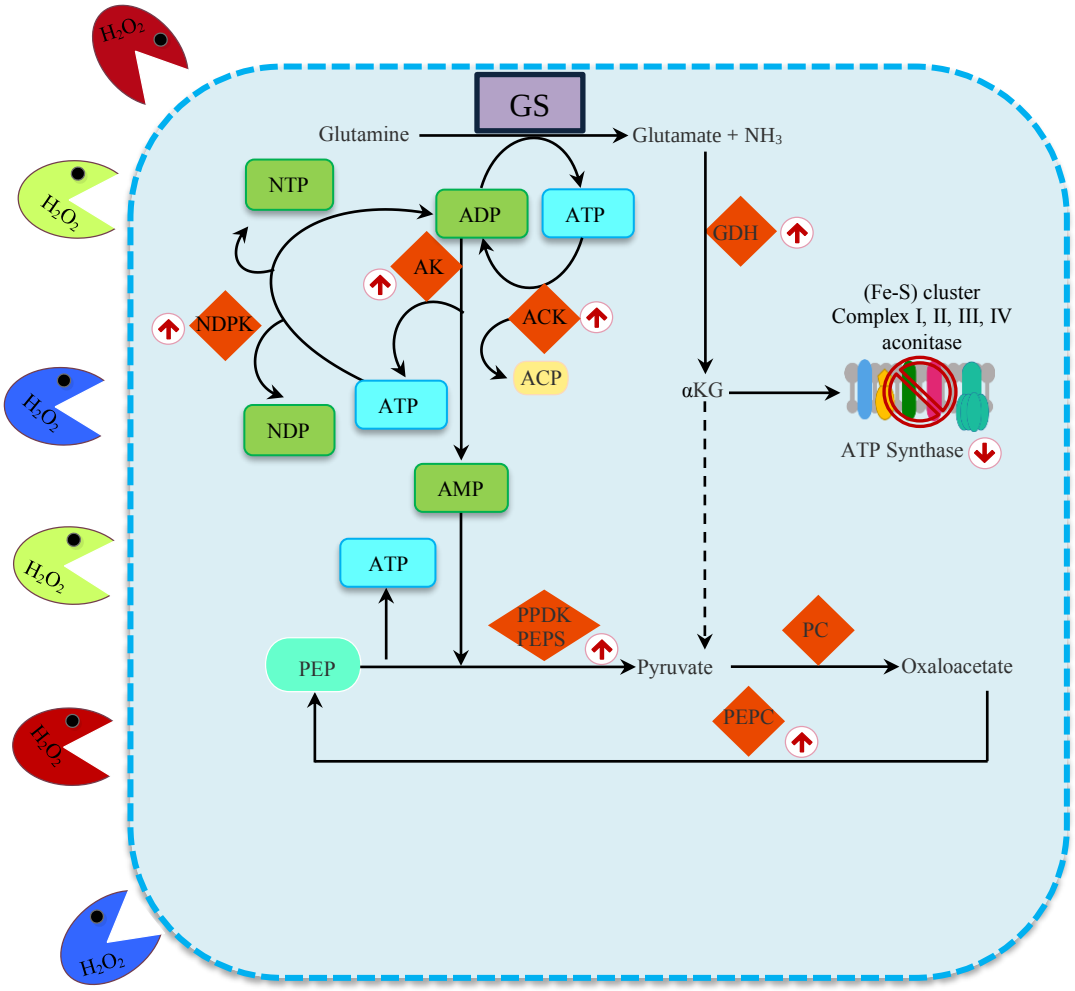

Figure 3.1-1 : A schematic representation of ATP homeostasis fueled by substrate-level phosphorylation during oxidative stress

3.2. Future work

This discovery of glutamine synthetase as a generator of energy under oxidative stress provides opportunities for further investigation to verify if amide bond can indeed be tapped into ATP. Asparagine another amide containing amino acid can be utilized as a source of carbon and nitrogen for *P.fluorescens* subjected to oxidative stress. Asparagine synthetase is an excellent candidate to release the locked energy in the amide bond of asparagine. In this instance, this enzyme that may liberate the ATP trapped in the amide bond can be studied. Findings from these investigations will establish the significance of amide bond as a source of energy in a manner analogous to thioester and phosphodiester containing compounds.

As glutamine is an important precursor to NADP biosynthesis, it will be interesting to evaluate if the production of this metabolite is enhanced during oxidative stress. NAD synthetase, a key conduit to NADP utilizes glutamine. This enzyme can be probed. The NAD formed is subsequently converted to NADP by NAD kinase. These two enzymes can act in partnership to shift production of these adenine nucleotides toward NADP (Figure 3.2-1). This situation may lead to enhanced NADPH formation, an anti-oxidant and the diminished synthesis of NADH, a pro-oxidant. Such a study may reveal a pivotal role of glutamine in combatting oxidative stress by switching on metabolic networks responsible for NADPH synthesis, a universal anti-oxidant.

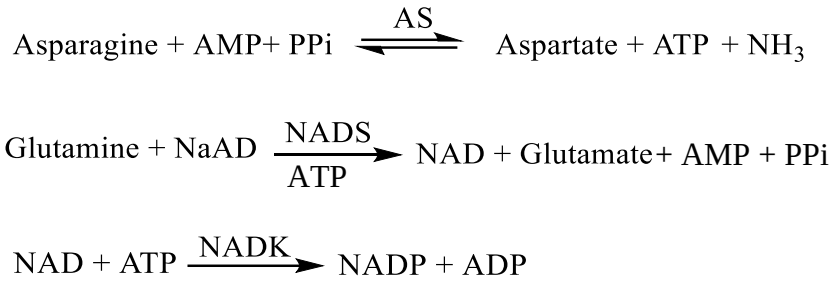

Figure 3.2-1: Future work on asparagine and NADP metabolism under oxidative stress. AS: Asparagine synthetase, NaAD: nicotinic acid adenine dinucleotide, NADS: nicotinamide adenine dinucleotide synthetase, NADK: nicotinamide adenine dinucleotide kinase.

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