In Silico Prediction of Phosphorylation Sites in Cu/Zn Superoxide Dismutase of Different Animals

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BY

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CERTIFICATE–I

*This is to certify that the thesis entitled, "**In Silico Prediction of Phosphorylation Sites in Cu/Zn Superoxide Dismutase of Different Animals**" submitted in partial fulfilment of the requirements for the award of the degree of Master of Science in ZOOLOGY to the Orissa university of Agriculture and technology, Bhubaneswar, is a faithful record of bonafide research work carried out by* Master ***JYOTI RANJAN CHOUDHURY****, Adm. No:09ZOL/14 under my guidance and supervision and that no part of thesis has been submitted for any other degree or diploma or published in any form.*

It is further certified that the help and sources of information availed of during the course of study have been duly acknowledged.

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CERTIFICATE–II

*This is to certify that the thesis entitled, "**In Silico Prediction of Phosphorylation Sites in Cu/Zn Superoxide Dismutase of Different Animals**" submitted by Master JYOTI RANJAN CHOUDHURY to the Orissa university of Agriculture and technology, Bhubaneswar, in partial fulfillment of the requirements for the degree of master of science in zoology, has been approved by the students' advisory committee after an oral examination on the same in collaboration with an External Examiner.*

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GLOSSARY

ATP- Adenosine Triphosphate

ADP- Adenosine Diphosphate

GTP- Guanosine Triphosphate

GDP- Guanosine Diphosphate

SH2- Src Homology 2

PTB- Phospho Tyrosine Binding domain

BRCT- BRCA1 C Terminus

ANT- ADP/ATP translocase

PDH- Pyruvate Dehydrogenase

GP- Glycogen Phosphorylase

PTMs- Post-translational modifications

PDHK- Pyruvate Dehydrogenase Kinase

PDHP- Pyruvate Dehydrogenase Phosphatase

AD- Alzheimer's disease

PD- Parkinson's disease

FTD-Fronto-temporal Dementia

ASyn- Alfa- synuclein

ROS- Reactive Oxygen Species

SOD- Superoxide Dismutase

Se-GPX- Selenium-Dependent Glutathione Peroxidase

GK- Greek key

PKC- Protein Kinase C

ML-III- Mucolipidosis III

ERK- Enzyme Regulated Kinase

CDK- Cyclin Dependent kinase

HSF- Heat-shock transcription factor

RD- Regulatory Domain

cAMP- Cyclin Adenosine Monophosphate

CREB- cAMP response element binding protein

HVC- High vocal center

STK33- Serine/ Threonine Kinase 33

MPF- Maturation promoting factor

CK- Creatine Kinase

CaMK- Calcium Kinase

AMPK- Adenosine Mono Phosphate Kinase

PKG- Protein Kinase G

TNF- Tumor Necrosis Factor

NLM- National Library of medicine

ABSTRACT

The discovery of superoxide dismutases (SODs), which convert superoxide radicals to molecular oxygen and hydrogen peroxide, has been termed the most important discovery of modern biology. Cu/Zn SOD is unanimously distributed in eukaryotes. Several reports have confirmed that Cu/Zn SOD has great significance in diverse biological courses, particularly anti-oxidant property of the cell. However, *DISPHOS* analysis of phosphorylation sites in the catalytic subunit of Cu/Zn SOD remains to be elucidated. I described the distribution of predicted (neural network predictions for serine (S), threonine (T) and tyrosine(Y)) STY phosphorylation sites in Cu/Zn SOD of 14 multicellular and 1 unicellular organisms. I also showed the clustering of Cu/Zn SOD in these organisms using a phylogram. This data showed the prevalence of Cu/Zn SOD with potential STY phosphorylation sites in several eukaryotic unicellular and multicellular organisms that could be significant in the context of enigmatic protein kinase activities of SOD, enzyme-substrate interactions, further ligand-binding studies and new therapeutic interventions.

Key words: Cu/Zn SOD, Protein phosphorylation sites, Phylogeny

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INTRODUCTION

CHAPTER-I

INTRODUCTION

What Is Phosphorylation?

Phosphorylation is the process through which a phosphate group (PO_4^{3-}) is transferred to a molecule, usually within a biological system. It is an important process within biological system. In biochemistry, a kinase is an enzyme that catalyzes the transfer of phosphate group(s) from high energy phosphate donating molecule to a specific substrate(s). This process is also known as phosphorylation, where, the substrate gains a phosphate group and the high energy adenosine triphosphate (ATP) molecule donates that phosphate group (Manning et al., 2002). In 1906, Phoebus Levene at the Rockefeller Institute for Medical Research identified phosphate in the protein vitellin (phosvitin), (Levene and Alsberg, 1906) and by 1933, had detected phosphoserine in casein, with Fritz Lipmann (Lipmann and Levene, 1932). However, it took another 20 years before Eugene P. Kennedy described the first 'enzymatic phosphorylation of proteins' (Burnett and Kennedy, 1954).

Types of Phosphorylation:

There are many types of phosphorylation. Some involve the transfer of phosphate to protein. Others consists of the production of ATP by phosphorylation of adenosine diphosphate (ADP). A third phosphorylation type helps to maintain the balance of sugar within the body and to promote metabolic processes. Mainly, there are 2 types of phosphorylation. These are as follows:

- Substrate level phosphorylation
- Oxidative phosphorylation

Substrate Level Phosphorylation:

Substrate level phosphorylation is a type of metabolic reaction that results in the formation of ATP or guanosine triphosphate (GTP) by the direct transfer and donation of a phosphoryl (PO_3) group to ADP or guanosine diphosphate (GDP) from a phosphorylated reactive intermediate. The main part of substrate-level phosphorylation occurs in the cytoplasm of cells as part of glycolysis and in mitochondria as part of the Krebs

cycle under both aerobic and anaerobic conditions. In the pay-off phase of glycolysis, two ATP are produced by substrate-level phosphorylation: two molecules of 1,3-bisphosphoglycerate are converted to 3-phosphoglycerate by transferring a phosphate group to ADP by a kinase; two phosphoenol-pyruvate are converted to pyruvate by the transfer of their phosphate groups to ADP by another kinase. Such a reaction takes place in the 5th step of the Krebs cycle. In this reaction, an enzyme known as succinyl-CoA synthetase facilitate the production of ATP from succinyl-CoA, inorganic phosphate, and ADP. The first step is the displacement of CoA by inorganic phosphate to form succinyl phosphate. The phosphate is removed, forming succinate and GTP. In the final step, the Phosphate is transferred to ADP to produce the high-energy ATP. Only 2 molecule of ATP are formed from each molecule of glucose (Fig. 1).

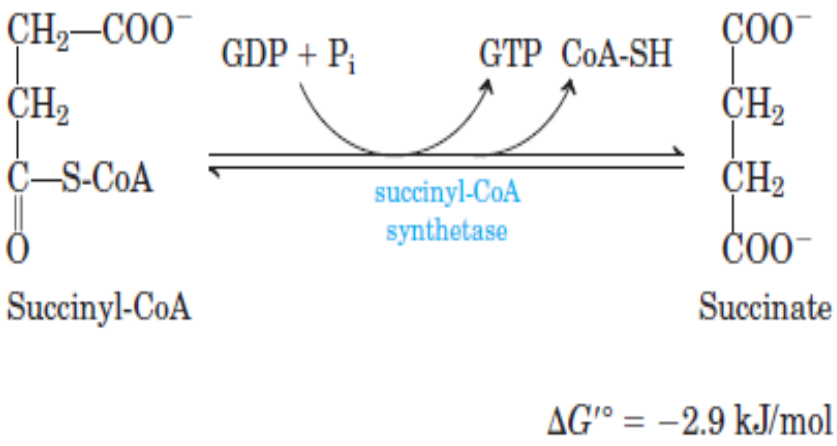

Figure 1. Substrate level phosphorylation shown in citric acid cycle

Oxidative Phosphorylation:

Oxidative phosphorylation is the culmination of energy-yielding metabolism in aerobic organisms. All oxidative steps in the degradation of carbohydrates, fats, and amino acids converge at this final stage of cellular respiration, in which the energy of oxidation drives the synthesis of ATP. Oxidative phosphorylation produces about 34 ATP, a basic events concerning oxidative phosphorylation, i.e. the synthesis of ATP at the expense of respired oxygen in mitochondria (Burnett, 1984). The oxidative phosphorylation system in mammalian mitochondria deals with: (i) the respiratory chain as a whole: redox centers of the chain and protonic coupling in oxidative phosphorylation (ii) atomic structure and functional mechanism of proton motive complexes I, III, IV and V of the oxidative phosphorylation system (iii)

biogenesis of oxidative phosphorylation complexes: mitochondrial import of nuclear encoded subunits, assembly of oxidative phosphorylation complexes, transcriptional factors controlling biogenesis of the complexes (Papa, 2012).

Protein Phosphorylation:

Protein phosphorylation is an important regulatory mechanism that occur in both prokaryotic and eukaryotic organisms (Cozzone, 1988).Phosphorylation usually occurs on serine, threonine, tyrosine and histidine residues in eukaryotic proteins but in case of prokaryotes, phosphorylation occurs in serine, threonine, tyrosine, histidine or arginine or lysine residues (Deutcher et al., 2005). In the 1950s, it was discovered that the molecular mechanism responsible for the conversion of the inactive state to the active state revealed that a protein kinase, i.e. phosphorylation kinase, could catalyse the attachment of phosphate to phosphorylase and render it fully active (Fischer et al., 1955).It was subsequently shown that phosphorylase kinase was itself activated by protein kinase and the concept of protein phosphorylation have been understood as the most physiologic process ranging from the cardiovascular system, gastrointestinal system, neurologic mechanisms and behaviour, immune response, endocrine action, musculoskeletal system regulation(Hunter, 2000). The concept of kinases began to take hold by the mid-1990s when the Abl tyrosine kinase inhibitor could affect dramatic remissions in more than 90% of preblast phase patients with chronic myelogenousleukemia (Capdeville, 2002). There are about 500 mammalian protein kinase, 100 protein phosphatases and hundreds of proteins containing domains (SH2, PTB, 14-3-3, BRCT, etc.) with interact with phosphorylated proteins (Yaffe, 2002). There are three chemical biological methods have been applied to sort out kinase action. (1) To modulate the action of kinase. (2) Specific site required to introduce phosphor-amino acids. (3) Fluorescent reporters that allow high resolution imaging of phosphoryl transfer in cells and lysates.

A Mechanism for the Evolution of Phosphorylation Sites

Protein phosphorylation is a ubiquitous mechanism for the temporal and spatial regulation of proteins involved in almost every cellular process. Protein phosphorylation is important in prokaryotes, where the best-characterized kinases catalyze the phosphorylation of histidine residues (Laub and Goulian, 2007). Phosphorylation appears to be even more important and more widespread in eukaryotic cells, but while histidine phosphorylation does

occur in at least some eukaryotes, most eukaryotic protein phosphorylation occurs at serine, threonine, and tyrosine residues (Manning et al., 2002a). The human genome includes about 500 genes for protein kinases that phosphorylate serine, threonine, and/or tyrosine residues (Manning et al., 2002b) and about 200 genes for phosphoprotein phosphatases that dephosphorylate them (Alonso et al., 2004). Thus, approximately 3.5% of human genes are devoted to proteins that directly regulate protein phosphorylation. The substrates of these kinases and phosphatases are numerous; one commonly cited estimate is that approximately 30% of proteins are phosphorylated (Cohen, 2000; Holt et al., 2009). Since many phosphor-proteins are phosphorylated at multiple sites, there are probably tens of thousands of different phosphorylation sites in the human proteome. Phosphorylation sites have been found in all of the isoforms of ANT (ADP/ATP translocase) by numerous investigators. These sites include, in ANT-1 (Ser7, Ser22, Tyr81, Thr84, Tyr191, and Tyr195) in ANT-2 (Thr84, Tyr191, and Tyr195) and in ANT-3 (Tyr112, Ser119, and Tyr195) (Boja et al., 2009).

Aerobes are pushed to oxidative stress (OS) when reactive oxygen species (ROS) are over produced in their cells. Superoxide anion radical ($O_2^{\bullet-}$), a potent ROS and the substrate of superoxide dismutase (SOD) enzymes is believed to be associated with several pathophysiologies due to its deleterious effects on biomolecules. Superoxide dismutase (SOD, EC 1.15.1.1) enzymes are the first line defence of enzymatic antioxidant defence which dismutates the toxic $O_2^{\bullet-}$ to less toxic H_2O_2 and H_2O molecule (DiDonato et al., 2003). Therefore, SOD enzymes have clinical application values including anti-inflammation, prevention of oncogenesis and tumor growth, protection against reperfusion and ischemic tissue etc. Besides therapeutic use, in environmental issues, studying SOD as bio-indicator in marine biotechnology has become an important area of environmental impact assessment. Over expressed human SOD has been reported to fight against environmental induced OS in yeast. Invertebrates do not have antibodies, but they posses some immune proteins belonging to the immunoglobulin super family which protect them against pathogens. In this regard, SOD contributes indirectly to increase the immunity to marine invertebrates by avoiding the internal $O_2^{\bullet-}$ anion toxicity and plays an important role in defending against radioactive toxicity and environmental mediated oxidative stress. Therefore, expression of SOD protein found to be used widely in monitoring pollution stress, pesticide effects, and disease indication, thermal and osmotic stress in invertebrates. SOD has also found to have relation with age and longevity of aquatic animals (DiDonato et al., 2003). All the above applications of SOD demand the need of studying its type and their properties especially with relation to interaction with their

inhibitors in different marine invertebrates. It will not be out of contest to mention here that depending on the prosthetic metals present in SOD enzyme active site, they are classified accordingly i.e. as Mn SOD, CuZn SOD, Fe SOD etc (DiDonato et al., 2003).

Objectives: Owing to the importance of phosphorylation and the enzyme CuZnSOD, the following objectives were set in the present study

- To identify the distribution of phosphorylated amino acids in CuZn SOD.
- To predict phosphorylation sites in CuZn SOD of different animals.
- To draw phylogenetic tree considering the sequences of SD among all studied organisms using Clustal Omega.

REVIEW OF LITERATURE

CHAPTER-II

REVIEW OF LITERATURE

Protein phosphorylation within the cardiac mitochondrial matrix and respiratory complexes is extensive. The dys-regulation of the cardiac energy conversion process has been suggested to be one of the majorelements in heart failure (Neubauer, 2007). Thus the precise orchestration of mitochondrial energy conversion is critical for normalheart function. Since enzyme proteinphosphorylation [i.e., pyruvate dehydrogenase (PDH) and glycogen phosphorylase (GP)] was one of the first mechanismof modulating metabolic processes to match physiological needs, it was logical to further explore enzyme proteinphosphorylation at other levels of energy metabolism of the heart. It should be pointed out that other post-translational modifications (PTMs) reviewed elsewhere, such as acetylation, nitration, and glutathionylation, have also been implicated in the regulation of mitochondrial metabolism.Since the late 1960s, protein phosphorylation was implicated as one of the regulatory mechanisms balancing energyconversion with utilization in the heart. Protein phosphorylation of mitochondrial PDH, described almost simultaneouslyby Linn et al. (1969) and Weiland and colleagues (Wieland et al., 1969), was one of the first examples of metabolic control byenzyme phosphorylation.

Many models of the regulation of cardiac mitochondrial oxidative phosphorylation suggest a distributed control of enzymatic activity. Therefore, it is a reasonable extrapolation, based on PDH and GP regulation, to look first at mitochondrial protein phosphorylation events that might contribute to metabolic regulation (Balaban, 2006). The critical communication between the mitochondrion and the cytosol across the outer mitochondrial membrane and the remarkably high-resistance inner membrane is dependent on numerous transporters. Thus modulation of these transporters via protein phosphorylation could dramatically affect the ability of the cytosol to influence mitochondrial reaction pathways via the exchange of metabolites and signalling molecules, as well as proteins (Zhou et al., 2007). The phosphorylation of PDH present in intermediary metabolism is via the PDH kinase (PDHK) and PDH phosphatase (PDHP) system is the first mitochondrial enzyme shown to be regulated by protein phosphorylation (Raul and Robert., 2012).

Protein misfolding and aggregation is common in neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD), fronto-temporal dementia (FTD). In these disorders, the misfolding and aggregation of specific proteins occurs alongside neuronal degeneration in somewhat specific brain areas, depending on the disorder and the

stage of the disease. In PD, alpha-synuclein (aSyn) forms protein aggregates, known as Lewy bodies, and is phosphorylated at serine 129. In AD and in FTD, hyperphosphorylation of tau protein causes its misfolding and aggregation (Frost and Diamond, 2010, Fig. 2).

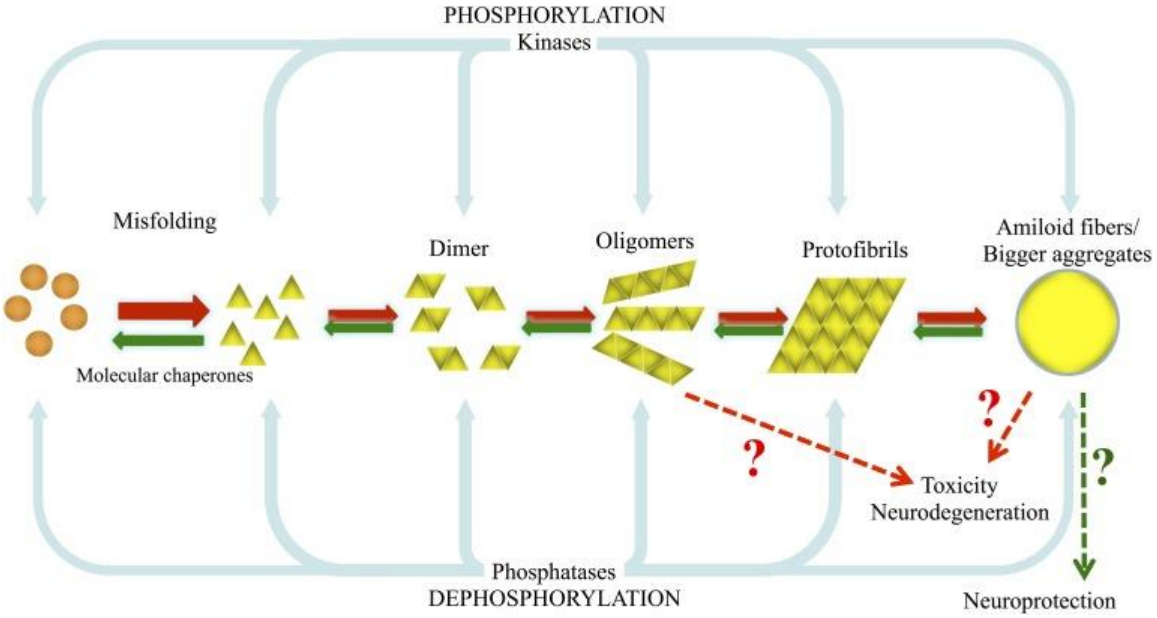

Figure 2. Model of a Syn and tau misfolding and aggregation, and the involvement of kinases and phosphatases on their phosphorylation/dephosphorylation

Studies on free radicals, antioxidants, and co-factors

One of the main driving force which helps human life to sustain are the biochemical reactions which take place within the organelles and cells of the body. The laws of nature are such that one moves from infancy to adulthood and finally one becomes a frail human being eventually leading to death. This aging process is a common feature of the life cycle of virtually all multicellular organisms. There are three main areas of research which are interlinked and can contribute or delay the aging process are; studies involving free radicals, antioxidants, and co-factors.

Free radicals

Free radicals can be defined as reactive chemical species having a single unpaired electron in an outer orbit (Riley, 1994). This unstable configuration creates energy which is released through reactions with adjacent molecules, such as proteins, lipids, carbohydrates, and nucleic acids (Riley, 1994). The majority of free radicals that damage biological systems are oxygen-free radicals, and these are more generally known as “reactive oxygen species” (ROS). ROS can be (i) generated during UV light irradiation and by X-rays and gamma rays (ii) produced during metal catalyzed reactions (iii) these are present in the atmosphere as pollutants

(iv) these are produced by neutrophils and macrophages during inflammation, and (iv) are by-products of mitochondrial catalyzed electron transport reactions, and various other mechanisms (Cadenas et al., 2000)

Antioxidants

The term "antioxidant" defined as any molecule capable of stabilizing or deactivating free radicals before they attack cells. Humans have evolved highly complex antioxidant systems, which work synergistically, and in combination with each other to protect the cells and organ systems of the body against free radical damage (Khalid, 2007) Antioxidants also work in both aqueous and membrane domains and effect gene expression in a positive way. There are several types of antioxidants present in our body. They are categorised in to 2 types i.e. enzymatic and non-enzymatic. Enzymatic antioxidants are glutathione peroxidase, catalase, and superoxide dismutase and non-enzymatic antioxidants are vitamin-E, Ascorbic acid, Melatonin, flavonoids etc.

Co-factors

The biochemical definition of a co-factor is that it is an ion or a molecule that binds to the catalytic site of an apo-enzyme rendering it active. Many enzymes have a requirement for metal ions for their activity and these metal ions are also referred to as co-factors. The major antioxidant enzymes possess transition metals or selenium at the catalytic site and the availability of cofactors can determine the activity of such enzymes. There is an example given showing metals act as a co-factor. Copper (Cu) is an essential cofactor in a number of critical enzymes including cytochrome C oxidase and copper, zinc-superoxide dismutase (Cu/Zn SOD). A Cu deficiency-induced decrease in the activity of CuZn-SOD in humans and animals has been reported (Turnlund et al., 1997). Copper deficiency also decreases the activity of ceruloplasmin, which requires Cu for its ferroxidase function and it can also lead to a reduction in enzymes of the oxidant defense system such as selenium-dependent glutathione peroxidase (Se-GPX) and catalase (Chen et al., 1994) Furthermore a deficiency in Cu can also alter other ROS scavengers including metallothionein (a Cu and Zn containing protein) and the non-protein thiol, glutathione (Adams and Keen, 2005). Copper and zinc are also essential co-factors for enzymes involved in the synthesis of various bone matrix constituents and could be important in the elderly since they may play an important role in reducing bone loss in osteoporosis (Lowe et al., 2002).

The structural biochemistry of superoxide dismutase

The discovery of superoxide dismutases (SODs), which convert superoxide radicals to molecular oxygen and hydrogen peroxide, has been termed the most important discovery of modern biology. SOD structures may also control their enzymatic activity through product inhibition; manipulation of these product inhibition levels has the potential to generate therapeutic forms of SOD. Markedly, structural destabilization of the SOD architecture can lead to disease, as mutations in Cu/Zn SOD may result in familial amyotrophic lateral sclerosis, a relatively common, rapidly progressing and fatal neurodegenerative disorder (Perry et al., 2009).

Three classes of SOD have evolved with distinct protein folds and different catalytic metal ions: the Cu,ZnSODs,MnSOD/FeSODs and NiSODs. Cu,ZnSOD (also known as SOD1 and SOD3 in humans) occurs in eukaryotes and some prokaryotes (DiDonato et al., 2003). Eukaryotic Cu,ZnSODs are highly conserved from the primary to quaternary structure. Cu,ZnSODs are composed of two identical subunits related by a two-fold symmetry axis. Each subunit consists of a β-barrel composed of eight antiparallel β-strands arranged in a Greek key motif (Richardson et al., 1977). Tightly packed hydrophobic residues form the core of the barrel, and loops β3/β4 and β6/β7 form the +3 β-strand Greek key connections (GK1 and GK2) (Tainer et al., 1982).

Two conserved Leu residues within these Greek key loops fill the ends of the β-barrel and are termed the cork residues. Two other major external loops form the active site channel. The first, the β4/β5 loop, tethers the dimer interface with the active site zinc and contains a stabilizing intervening disulfide bond that aids stability (Getzoff et al., 1989). The disulfide stabilizes both the subunit fold and the dimer interface. The second, β7/β8 or electrostatic loop (EL), guides and accelerates the substrate O_2^- into the active site (Getzoff et al., 1992).The active site in each Cu/ZnSOD subunit contains one Cu ion ligated by three histidines when in the reduced state and one Zn ion ligated by one aspartic acid and three histidines, whose side chains all reside outside of the β-barrel. One of the histidine ligands of the Zn ion ligands also ligates the Cu ion when in the oxidized state and thus has been termed the bridging histidine (Strange et al., 2006).Both the β-barrel fold and a tight hydrophobic dimer interface provide structural stability to Cu/ZnSOD. The hydrophobic β-barrel core and main-chain β-sheet hydrogen bonds were shown to be key in promoting not only structural integrity, but also folding, as a set of circular permutation mutants with swapped connections of the inter-β-strand loops and N- and C-termini yielded active enzymes. Both the binding of the active site metal ions and formation of the conserved disulphide bond in each subunit also contribute to the

framework stability and specificity of the protein fold and dimer assembly (Boissinot et al., 1997).

Ser/Thr/Tyr Protein Phosphorylation in Bacteria

Ser/Thr/Tyr phosphorylation of a bacterial protein was first clearly established in isocitrate dehydrogenase. This enzyme was reported to become phosphorylated on a serine residue in1979, 25 years after the discovery of protein phosphorylation in eukaryotes. Numerous other bacterial proteins phosphorylated on Ser, Thr or Tyr were discovered and the corresponding protein kinases and P-protein phosphatases were identified in subsequent years. These protein modifications regulate all kinds of physiological processes. Therefore, Ser/Thr/Tyr phosphorylation in bacteria seems to play a similar important role as in eukaryotes. Thus it was observed that many bacterial protein kinases do not exhibit any similarity to eukaryotic protein kinases, but rather resemble nucleotide-binding proteins or kinases phosphorylating diverse low-molecular-weight substrates (Deutscher and saier, 2006).

Genetic and biochemical studies on protein phosphorylation in the circadian clock of Drosophila melanogaster

In almost all phyla circadian rhythms in physiology and behavior are observed. Such rhythms are generated by genetically encoded internal clocks. For the generation and maintenance of endogenous circadian near 24h rhythms there is requirement of gene products which has led to a paradigm of multiple interlocked transcriptional/translational feedback loops as the basis for molecular circadian oscillators in all studied model systems. Protein phosphorylation plays an essential role, regulating the stability, activity and subcellular localization of proteins that constitute the biological clock (Kivimae, 2005). Investigation of the role of the protein kinase Doubletime, a Drosophila ortholog of caesin kinase in its circadian clock was done. Production of the enzymatically active Doubletime protein and direct phosphorylation of clock protein period was demonstrated for the 1st time. In a cell culture system test for phosphorylation sites identification and significance of functional significance was done. Period mutant that eliminates one of the identified phosphorylation sites is also carried out and ti was known from in vivo analysis. Thus, phosphorylation dependent regulation of Period protein stability,transcriptional repressor activity and possibly subcellular

localization may all be regulated in an integrated fashion that involves two sequence motifs in the center of the Period protein with high affinity for phosphorylation by Doubletime (Kennedy, 2015).

Catch muscle myorod modulates ATPase of myosin in a phosphorylation-dependent way

In molluscan catch muscle myorod is exclusively expressed. It localises on the surface of thick filaments together with twichin and myosin. An alternatively spliced product of the myosin heavy chain is myorod that contains C-terminal rod part of myosin and a unique N-terminal domain. The target for phosphorylation is the unique domain by gizzard smooth myosin light chain kinase (smMLCK) and, perhaps, molluscan twichin containing a MLCK like domains. For elucidating the role of myorod and its phosphorylation in the catch muscle the effect of chromatographically purified myorod on the actin-activated $Mg2^{+}$-ATPase activity of myosin was studied. It was found out that at the N-terminus of myorod potentiated the actin-activated $Mg2^{+}$-ATPase activity of mussel myosin. Thus, myorod could be related to the catch state, a function specific to molluscan muscle (Matusovsky et al., 2015).

A requirement for protein phosphorylation in regulating the meiotic and mitotic cell cycles in echinoderms

Synchronous meiotic and mitotic divisions are observed in populations of hormone-stimulated starfish oocytes and fertilized sea urchin eggs. The requirement for protein phosphorylation during these events by testing the effects of 6-dimethylaminopurine (6-DMAP) upon the incorporation of [^{32}P] orthophosphate was studied. It was found that 6-DMAP blocked meiosis reinitiation and early cleavage and simultaneous inhibition of protein phosphorylation without changing the rate of [^{35}S] methionine incorporation or pattern of protein synthesis occurred. Synthesis of the protein, cyclin (54 kDa in starfish and 57 kDa in sea urchin) contined in the presence of 6-DMAP. First destruction of this protein occurs and then second cell cycles when 6-DMAP is added 30 min following fertilization. Thus, cyclin breakdown does not depend on the completion of the nuclear events of M-phase. Its time of breakdown is set at an early step between fertilization and first cleavage. 6-DMAP did not affect the cortical microtubules and resting female centrioles of prophase-arrested starfish oocytes whereas it induced a precocious disappearance of spindle fibers when applied to hormone-stimulated oocytes was known by using tubulin immunostaining. A late treatment always allowed chromosome separation and centriole separation and thus pericentriolar tubulin

persisted and could organize new spindles after the inhibitor was removed. Thus, the assembly of cortical and centriolar-associated microtubules is not controlled by the same factors as spindle-associated tubulin. Specific proteins which are required for the cell to enter the following M-phase can become operative only via a process depending upon protein phosphorylation and microtubule-associated kinases may play an important role in MPF function and spindle dynamics (Neant et al., 1989).

Proteasomes regulate the motility of salmonid fish sperm through modulation of cAMP-dependent phosphorylation of an outer arm dynein light chain

Regulation of sperm motility in salmonid fish is ATP dependent involving proteasomes. Immunoelectron microscopy demonstrated that proteasomes are located at the structure of the chum salmon sperm flagellum that attaches at the base of the outer arm dyenin and extends towards the plama membrane. In chum salmon sperm inhibition of cAMP dependent phosphorylation of a 22 kDa anoxemal protein is done by substrates and inhibitors of proteasomes. Treatment of the anoxeme with a high salt solution and subsequent sucrose density gradient centrifugation of the extract which solubilised the 22kDa phosphoprotein also revealed with 19S outer arm dynein, indicating that it is a dynein light chain. Thus proteasomes modulate the activity of outer arm dynein by regulating cAMP dependent phosphorylation of the 22kDa dynein light chain (Inaba, 1998).

Creatine kinase regulation by reversible phosphorylation in frog muscle

From skeletal muscle of wood frogs, Ranasylvatica, a species that survives natural whole body freezing during the winter month's creatine kinase (CK) was analyzed. It was observed that when frogs froze, muscle CK activity increased by 35%and apparent Kmcreatine decreased by 29%. Increase in this activity was not due to a change in total CK protein was observed in immuno-blotting analysis. Frog muscle CK was regulated by reversible protein phosphorylation. Conditions that facilitated the actions of various protein kinases (PKA, PKG, PKC, CaMK or AMPK) along with in vitro incubations with 32P-ATP resulted in immuno-precipitation of 32P-labeled CK. Incubations that stimulated CaMK or AMPK altered CK kinetics. Incubation under conditions that facilitated protein phosphatases (PP2B or PP2C) reversed these effects. CK increased activity due to phosphorylation, whereas decreased due to dephosphorylation. Two forms of CK with different phosphorylation states were present in

muscle; low versus high phosphate forms dominated in muscle of control versus frozen frogs, respectively was revealed from Ion-exchange chromatography. CK from control versus frozen frogs showed no differences in susceptibility to urea denaturation or sensitivity to limited proteolysis by thermolysin. Thus increased activity, increased substrate affinity and altered phosphorylation state of CK in skeletal muscle from frozen frogs argues for altered regulation of CK under energy stress in ischemic frozen muscle (Deini and Storey, 2009).

Changes in protein phosphorylation accompanying maturation of Xenopuslaevis

After injection of phosphate into oocytes of *Xenopus laevis* which was undergoing progesterone induced meiotic maturation, protein phosphorylation was measured. After progesterone exposure and shortly before germinal vesicle break down, as the oocytes mature there is a burst of non-yolk protein phosphorylation. This was not due to changes in the specific activity of phosphate or ATP pool. Enucleated oocytes also experience burst as there is cytoplasmic location of phosphoprotein formation. After the injection of cytoplam containing maturation promoting factor (MPF) to the oocytes a burst of protein phosphorylation occurs immediately along with occurrence of GVBD even in the presence of cycloheximide. Oocytes which undergo GVBD are the only ones to have experienced the phosorylation burst. Thus the protein phosphorylation burst is a necessary step in the mechanism by which MPF promotes GVBD (Maller et al., 1997).

Reptilian uncoupling protein: functionality and expression in sub-zero temperatures

The partial nucleotide sequence of a reptilian uncoupling protein (repUCP) gene was taken from the European common lizard (*Lacerta vivipara*). Protein shows 55%, 72% and 77% sequence homology with rat UCP1, UCP2 and UCP3. Detection of repUCP gene in 4ºC cold-acclinated lizard tissues and up-regulated in muscle tissues by a 20h exposure to sub-zero temperature in a supercooling state or after thawing as it was revealed in overlapping sequence analysis. An increase in the co-activators, peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and peroxisome proliferator-activator receptor (PPAR), mRNA expression was observed suggesting that the mechanism regulating UCP expression may be conserved between mammals and reptiles. A quanosine diphosphate (GDP) sensitive non phosphorylating respiration was observed in mitochondria extracted from lizard skeletal

muscle. It indicates an inhibition of extra proton leakage mediated by an uncoupling protein providing arguments that repUCP is functional in lizard tissues. Thus, this is associated with a remarkable GDP-dependent increase in mitochondrial endogenous H2O2 production. It supports a physiological role of the repUCP in superoxide limitation by lizard mitochondria in situation of stressful oxidative reperfusion following a re-waming period in winter (Rey, 2008).

17β estradiol induces spermatogonial proliferation through mitogen activated protein kinase activity in the lizard

For normal male fertility, 17β estradiol (E2) is necessary. A non-mammalian vertebrate model (lizard) is used to investigate the regulation of extracellular signal regulated kinase 1 and 2 (ERK1/2) activity in the testis during the annual sexual cycle and studied whether E2 exerted a role in the spermatogenesis through ERK1/2 activity.ERK1/2 proteins are present in the nucleus of the spermatogonia (SPG) and primary spermatocytes (SPC) was observed in immunocytochemistry analysis. A progressive increase during the active spermatogenesis and a peak in the month of August was shown in the annual profile. During the period of active spermatogenesis and in post refractory period compared with winter stains in parallel ERK1/2 are highly phosphorylated. Thus, E2 treatment induces spermatogonial proliferation, possibly via the activation of ERK1/2, and this effect is counteracted by the antiestrogen (Chieffi et al., 2002).

Metabolic adaptations supporting anoxia tolerance in reptiles

Survival of animals during severe hypoxia and/or anoxia is enhanced by a variety of biochemical adaptations including adaptations of fermentative pathways of energy production. Most important is the ability to sharply reduce metabolic rate by 5-20 fold and enter a hypometabolic state. Prove to have common molecular principles that extend across phylogenetic lines and that are conserved in different types of arrested states is the biochemical regulation of metabolic arrest. Using the models like freshwater turtle Trachemys scripts elegans the new studies with anoxia tolerant vertebrates have identify a variety of regulatory mechanisms involved in both metabolic rate depression and in the aerobic recovery process. Mechanisms include the post-translational modification of cellular and functional proteins by reversible phosphorylation and changes in protein kinase and/or phosphatase activities to regulate this. It includes reversible enzyme binding association with subcellular structural element. It also includes differential gene expression and/or mRNA translation producing new

mRNA variants and new protein products. It causes changes in protease activity, particularly the multicatalytic proteinase complex. Mechanisms involve both constitutive and anoxia induced modification to cellular antioxidant system to deal with oxidative stress during the anoxic aerobic transition of recovery (Storey, 1996).

Behavioural effects of brain-derived estrogens in birds

Estrogens produced in the brain by aromatization of testosterone have widespread effects on behaviour in birds as in other vertebrates. Researches on male Japanese quail demonstrates that effects of brain estrogens on all aspects of sexual behaviour including appetite and consumatory components as well as learned aspects. This can be divided into 2 main classes based on their time course. First, the transcription of a variety of genes involved primarily in neurotransmission is regulated by estrogens via binding to estrogen receptors. Thus, results in activation of male copulatory behaviour after a latency of few days. There is increase in transcription of the aromatase mRNA by the testosterone and its aromatized metabolites. Thus, this results in an increased concentration and activity of the enzyme that actually precedes behavioural activation. Second, recent studies reveal that phosphorylation can modulate within minutes the brain aromatase activity. This is regulated by changes in intracellular Ca^{++} concentration such as those associated with glutamatergic neurotransmission. Rapid up or down regulation of brain estrogen concentration presumably resulted from these changes in AA affect, by non-genomic mechanisms with relatively short latencies. Thus, brain estrogens affect behaviour on different time scales by genomic and non-genomic mechanisms similar to those of a hormone or a neurotransmitter (Balthazart et al., 2015).

Phosphorylation of nuclear protein in avian erythrocytes

In nuclear phosphorylation changes have been followed during the course of development of the avian erythrocyte. Both young reticulocytes and mature erythrocytes are capable of incorporating 33Pi into nuclear proteins (Kleinsmith, 1969). The bulk of the activity occurring in the non-histone protein fraction. In the younger cells, both phosphorylation and dephosphorylation of these proteins occur at faster rates. The nuclear levels of both phosphorylation kinase and protein bound phosphorous fall several fold as maturation proceeds. Thus changes in the metabolism and composition of these nuclear phospho-proteins correlate with changes in the structure and activity of the nucleus (Gershey, 1971).

Song induced phosphorylation of cAMP response element binding protein in the songbird brain

Investigation in the participation of cAMP response element binding protein (CREB) in the response of the songbird brain to a natural auditory stimulus, is a conspecific song. There was a intense staining with an anti-CREB monoclonal antibody of two song control nuclei is the higher vocal center (HVC) and area X of zebra finches (Taeniopygiaguttata). Double labelling studies showed that CREB immune reactively was detected only in area X-propelling neurons in HVC. The cloned CREB cDNA from zebra finches (zCREB) is homologous to mammalian ΔCREB. Hearing tape recorded conspecific songs of zebra finches induces phosphorylation zCREB at Ser119 in area X projecting HVC neurons. Thus, there is rise in possibility that zCREB plays a crucial role in the sensory process of song learning (Sakagnuchi et al., 1999). Conservation of serine/threonine kinase 33 (STK33) across all major vertebrate classes including reptiles, mammals, amphibians and fish, suggest its importance within vertebrates. It has been shown to phosphorylate vimentin and might play a role in spermatogenesis and organ ontogenesis. Using a combination of large scale next generation sequencing data analysis and traditional PCR there was analysis of the genomic locus and expression of *stk33* in the class Aves (Lautwein et al., 2015).

Phospholipid turnover as a possible trans-membrane signal for proteins in mammals

A large volume of Ca2+ activated, phospholipid-dependent protein kinase C (protein kinase C) is present in human platelets. Hydrolysis of thrombin induced phosphatidyl inositol resulted in activation of this enzyme which is initiated by unsaturated diacylglycerol. In vitro phosphorylation of PKC occurs having a molecular weight of about 40,000(40 K protein). Thrombin as well as by exogenous phospholipase C stimulates the rapid labelling of this protein in platelets. Diacylglycerol formation always accompanies 40K protein phosphorylation. Induction of the phosphorylation of 40K protein in vivo is selectively inhibited by chlorpromazine and dibucaine. Thus phosphatidylinositol turnover provoked by thrombin seems to serve as a trans-membrane signal for protein phosphorylation during platelet activation (Kawahara et al., 1980).The enzyme uridine diphospho-N-acetyl-glucosamine lysosomal enzyme N-acetylglucosamine-1-phosphotransferase cause the lysosomal storage disease mucolipidosis III (MLIII) which is the primary genetic disease. This enzyme conducts two functions specific recognition of lysosomal enzymes (recognition functions) and

phosphorylation of their oligosaccharides (catalytic functions). Fibroblasts were taken from patients with MLIII as the source of enzyme and α-methylmannoside and two lysosomal enzymes as the substrates. Thus, there was identification of defects in both of these functions. In one group of fibroblasts, the catalytic activity of the N-acetylglucosaminylphosphotransferase is decreased while the ability to recognise lysosomal enzymes as specific substrates remains intact (Lang et al., 1985).

Mammal-specific, ERK-dependent, Caldesmon Phosphorylation in Smooth Muscle

During smooth muscle stimulation, extracellular signal-regulated kinases (ERKs) phosphorylates the high molecular mass isoform of the actin-binding protein caldesmon (h-CaD) at two sites (Ser759 and Ser780). Antibodies were generated against phosphopeptides analogus to the sequences around these two sites. In porcine carotid arterial muscle strips, the major site of phosphorylation in h-CaD was at Ser789. Phosphorylation at Ser759 of h-CaD was undetectable. Phosphorylation of the low molecular mass isoform of the protein (l-CaD) at the site analogous to Ser789 was greater in serum-stimulated cultured smooth muscle cells than in serum-starved cells (Angelo, 1999). Serum-stimulated l-CaD phosphorylation was attenuated by the protein kinase inhibitor PD98059. Thus there was identification of Ser789 of h-CaD as the major site of ERK-dependent phosphorylation in carotid arteries. It was observed that the level of phosphorylation at Ser789 is relatively constant following carotid arterial muscle stimulation, despite an increase in total protein phosphate content; and suggested a functional role for ERK-dependent l-CaD phosphorylation in cell division.

Src kinase regulation by phosphorylation and dephosphorylation

The regulatory protein Src and Src family protein tyrosine kinases play important roles in cell differentiation, motility, proliferation, and survival. Auto phosphorylation produces an initially described phosphorylation sites of Src which includes an activating phosphor-tyrosine 416. Phosphorylation by C-terminal Src kinase (Csk) and Csk homologous kinase resulted in an inhibiting phosphotyrosine 527. Src kinase activity increases by dephosphorylation of phosphotyrosine 527. The phosphorylation of SrcTyr 138 is mediated by the platelet-derived growth factor receptor protein tyrosine. This phosphorylation has no direct effect on Src kinase activity. Phosphorylation of SrcTyr 213 and activation of Src kinase activity is mediated by the platelet-derived growth factor receptor and the ErbB2/HER2 growth factor receptor protein tyrosine kinases. The substrate for protein serine/threonine kinases including protein kinase

(Ser-12), protein kinase A (Ser-17) and CDK1/cdc2(Thr 34, Thr 46 and Ser 72) is Src kinase.Out of these, phosphorylation by CDK1/cdc2 has been demonstrated to increase Src kinase activity. The nature of phosphoprotein phosphatases that catalyzes the hydrolysis of phosphotyrosine 527 is known but of phosphatases of phosphotyrosine 138 and 213 and phosphoserine and phosphothreonine is not determined (Roskoski, 2005).

Cu/Zn Superoxide Dismutase plays important role in immune response

The role of Cu/Zn superoxide dismutase (SOD-1), an important enzyme in cellular oxygen metabolism, was examined in activated peritoneal elicited macrophages (PEM) and in several inflammatory processes in-vivo. LPS and TNF-α induced SOD-1 in PEM. SOD-1 induction by LPS was mainly via extracellular signal-regulated kinase-1 activation. Transgenic mice overexpressing SOD-1 demonstrated a significant increase in the release of TNF-α and of the metallo-proteinases MMP-2 and MMP-9 from PEM. Disulfiram (DSF), an inhibitor of SOD-1, strongly inhibited the release of TNF-α, vascular endothelial growth factor, and MMP-2 and MMP-9 from cultured activated PEM. These effects were prevented by addition of antioxidants (Marikovsky et al., 2003).

Oxygen radicals are produced as by-products of normal oxidative metabolism. Hence, activated cells with increased metabolism produce more oxygen radicals and macrophages, which are phagocytic cells, produce and release reactive oxygen species (ROS) in response to phagocytosis or stimulation with various agents (Malmstrom, 1982). It has long been known that control of the intracellular redox environment is vital for proper cellular function. To protect themselves from the constant oxidative challenge, cells have developed defence mechanisms that ensure a proper balance between pro- and antioxidant molecules (Forman and Torres, 2001). Cu/Zn superoxide dismutase (SOD) is a key enzyme in the dismutation of superoxide radicals resulting from cellular oxidative metabolism into hydrogen peroxide (H_2O_2). Because inflammation is characterized by macrophage activation, the possibility that altered SOD activity would affect the inflammatory process (Fridovich, 1978).We speculated that up-regulation of SOD would increase the ability of macrophages to confront an increased level of ROS during inflammation, resulting in increased immune response. In contrast, inhibition of SOD would diminish this ability, resulting in inhibition of the immune response. (Forman et al., 1980).Disulfiram (DSF), inhibits SOD-1 which induces oxidative stress and apoptosis in endothelial cells. LPS arrests macrophage proliferation and activates them to produce pro-inflammatory factors such as arachidonic acid metabolites, nitrogen intermediates, and cytokines, such as IL-1, IL-6, and TNF-α, which play important roles in the immune

response (Adams and Hamilton, 1984). TNF-α possesses bioactivities that are important in regulating the inflammatory response, including: inducing expression of adhesion molecules (Collins et al., 1995) and stimulating production of other inflammatory molecules, including IL-1, IL-6, platelet-derived growth factor, TGF, and arachidonic acid metabolites such as PGE2 and prostacyclin. TNF-α also stimulates the production of reactive oxygen and nitrogen species by leukocytes (Adler et al., 1994). Early induction of TNF-α therefore initiates a cascade of responses that contribute to the recruitment and activation of inflammatory cells and immune reactions.

MATERIALS AND METHODS

MATERIALS AND METHODS:

The following online tools and servers were used to achieve the objectives.

Pubmed Central

PubMed central is a free digital database of full-text scientific literature in biomedical and life sciences (Fig. 3). It grew from the online Enterz PubMed biomedical literature search system. PubMed Central was developed by the U.S. National Library of medicine (NLM) as an online archive of biomedical journal articles. The full text of all PubMed Central articles is free to read, with varying provisions for reuse. Some participating publishers delay the release of their articles on PubMed Central for a set time after paper publication (often 6 months). As of January 2016, the archive contains approximately 3.9 million items, including articles, editorials, and letters. It appears to be growing by at least 7% per year.

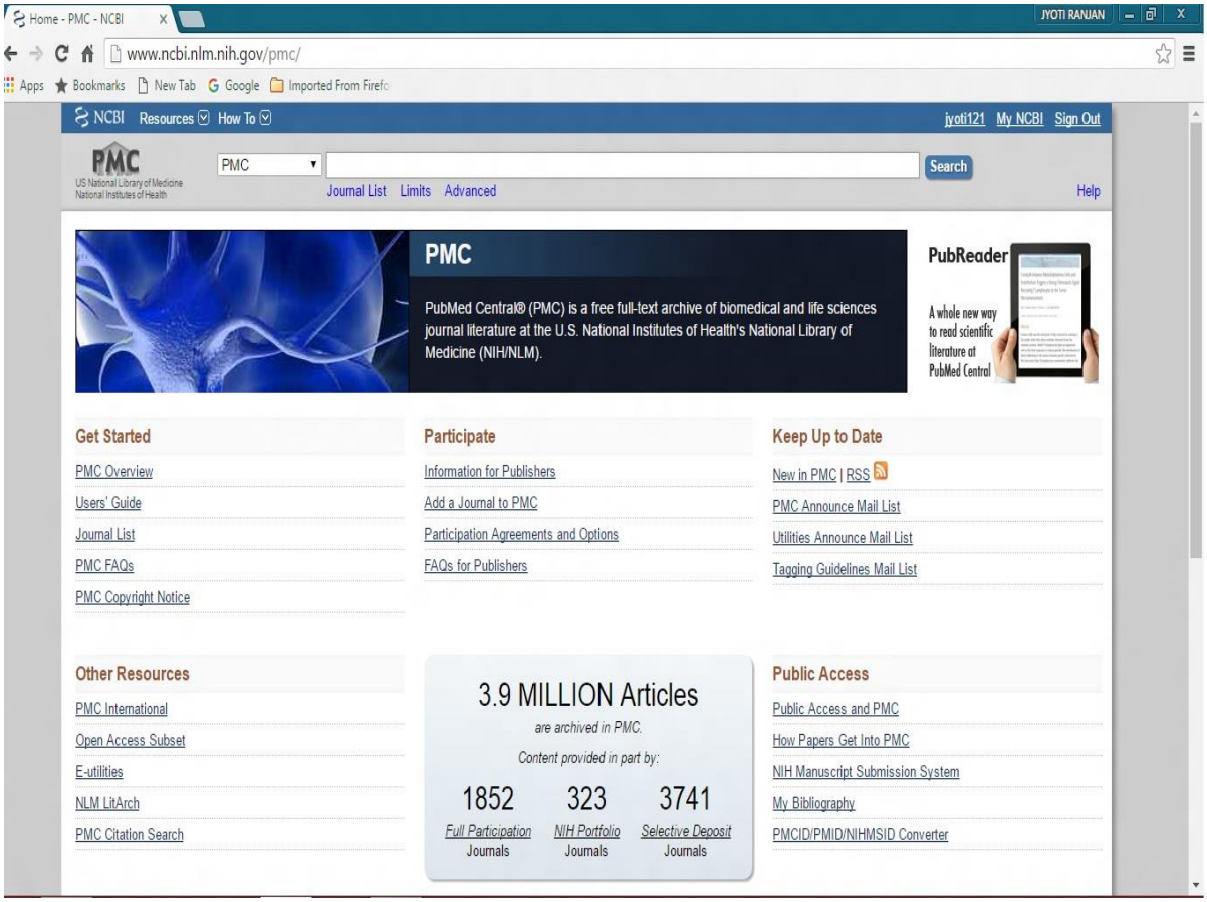

Figure 3. Home page of PubMed Central

Clustal Omega

At the European Bioinformatics Institute (EMBL-EBI), we help scientists realise the potential of 'big data' in biology, helping them exploit complex information to make discoveries that benefit mankind. We manage the world's public biological data and make it freely available to the scientific community via a range of services and tools, perform basic research and provide professional training in bioinformatics. Clustal Omega is a program which was developed for alignment of multiple sequences then after click the submit button it give the aligned sequence from which we make the phylogenetic tree by using protein sequences (Fig. 4).

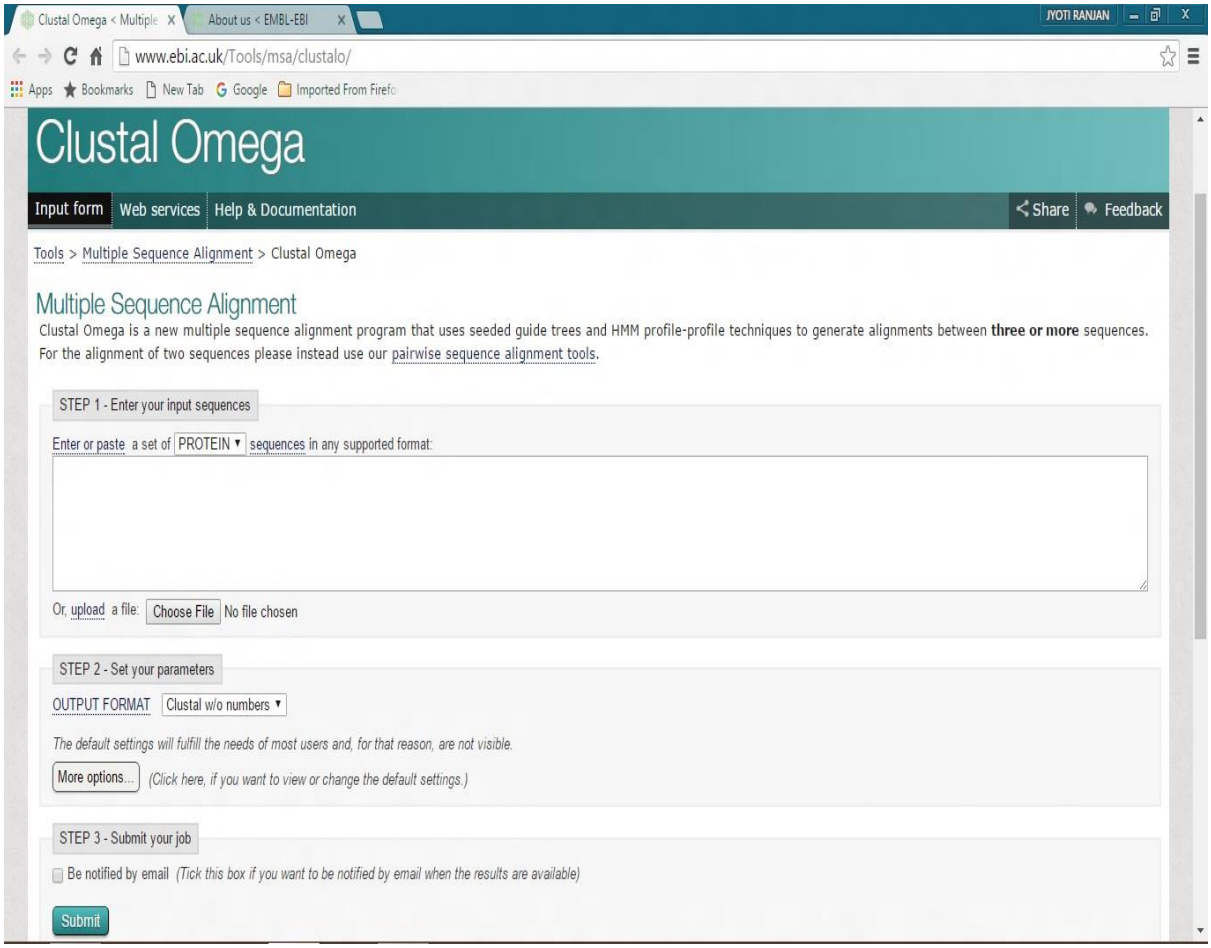

Figure 4. Home page of Clustal Omega

CuZn superoxide dismutase (SOD) protein sequences were obtained from GenBank. The dataset consists of CuZn SOD from 14 multicellular organisms and 1 unicellular organism (Table 1).

Table 1: Cu/Zn SOD subunit sequence dataset from GenBank (release 184.0, June 2011)

Genbank accession	Organisms
4507149|ref|NP_000445.1|	*Homo sapiens*
8394328|ref|NP_058746.1|	*Rattus norvegicus*
45597447|ref|NP_035564.1|	*Mus musculus*
45384218|ref|NP_990395.1|	*Gallus gallus*
528078368|ref|NP_001268469.1|	*Melopsittacus undulates*
406829601|gb|AFS63893.1|	*Thamnophis elegans*
147906753|ref|NP_001080933.1|	*Xenopus laevis*
56790262|ref|NP_571369.1|	*Danio rerio*
330317796|gb|AEC11112.1|	*Cyprinus carpio*
451844586|gb|AGF70691.1|	*Apostichopus japonicus*
34481600|emb|CAE46443.1|	*Mytilus edulis*
17136496|ref|NP_476735.1|	*Drosophila melanogaster*
62005086|gb|AAX59897.1|	*Bombus ignites*
211970334|emb|CAR97839.1|	*Caenorhabditis elegans*
51243303|gb|AAT99430.1|	*Saccharomyces cerevisiae*

Phosphorylation sites in CuZn SOD werelocated using the DISPHOS 1.3 server. It is a predictionserver for predicting probable phosphorylation sites atserine, threonine and tyrosine residues in protein by anartificial neural network method in independentsequences with sensitivity.

About DISPHOS

DISPHOS computationally predicts serine, threonine and tyrosine phosphorylation sites in proteins. The new version of the predictor (DISPHOS 1.3) was trained on over 2000 non-redundant experimentally confirmed protein phosphorylation sites (1,079 Serine sites, 666 Threonine sites, and 375 Tyrosine sites). The new set of phosphorylation sites was augmented using the entries from SwissProt R44, Phospho. ELM database, and literature. The observation that amino acid composition, sequence complexity, hydrophobicity, charge and other sequence attributes of regions adjacent to phosphorylation sites are very similar to those of intrinsically disordered protein regions suggests that disorder in and around the potential phosphorylation target site is an important prerequisite for phosphorylation. Thus, DISPHOS uses disorder

information to improve the discrimination between phosphorylation and non-phosphorylation sites. The accuracy of DISPHOS reaches 81.3% +/- 2.2% for Serine, 74.8% +/- 2.5% for Threonine, and 79.0% +/- 2.4% for Tyrosine. The application of DISPHOS to ordered and disordered protein regions, as well as to various functional protein categories and proteomes provides strong support for the hypothesis that protein phosphorylation predominantly occurs in regions of intrinsic disorder.

Usage of DISPHOS

Input

First paste your query sequence in FASTA format (only 20 symbols corresponding to the conventional amino acid code are supported). Then choose the kingdom from which the protein originates. If it is not known, please check "unknown" box. If your protein is eukaryotic and you know the organism, from which it originates, please check the appropriate boxes. If the organism is unknown, then leave the field blank. If you know the functional category, to which your query protein belongs, then check the appropriate "functional category" box. If the category is unknown or cannot be found in the list provided, please leave the field blank. Although DISPHOS was trained on eukaryotic phosphorylation sites, it was applied to estimate phosphorylation rates in different proteomes and protein functional categories to achieve a better precision. These estimates were incorporated into predictor to allow for prediction on organisms other than eukaryotes (Fig. 5).

Output

The output consists of 5 columns: position, residue, phosphorylation score, surrounding sequence, phosphorylation annotation (yes for positive result). The predictions are made on all serine, threonine and tyrosine residues of a query sequence. Only residues with the score >0.5 are considered to be phosphorylated. A high score implies a more confident positive prediction. Graphical output shows only residues that are predicted to be phosphorylated (DISPHOS score >0.5). The score generally approximates the probability that the residue is phosphorylated, given the information about the protein sequence (such as kingdom, organism and functional category).

Outputs are adjusted to the estimated class priors (relative frequencies of non-phosphorylated and phosphorylated residues) of each group. Hence, the predictor will try to

decrease the number of misclassified residues. Note that since the probability that a site is phosphorylated is usually smaller than 0.5 (except for some functional categories), this will typically reduce the number of sites predicted to be phosphorylated. If there is no information about the sequence or the user needs to know to which class the new query site is closer (P- or NP- class) the Default Predictor should be used (assumed class priors are 0.5 and 0.5).

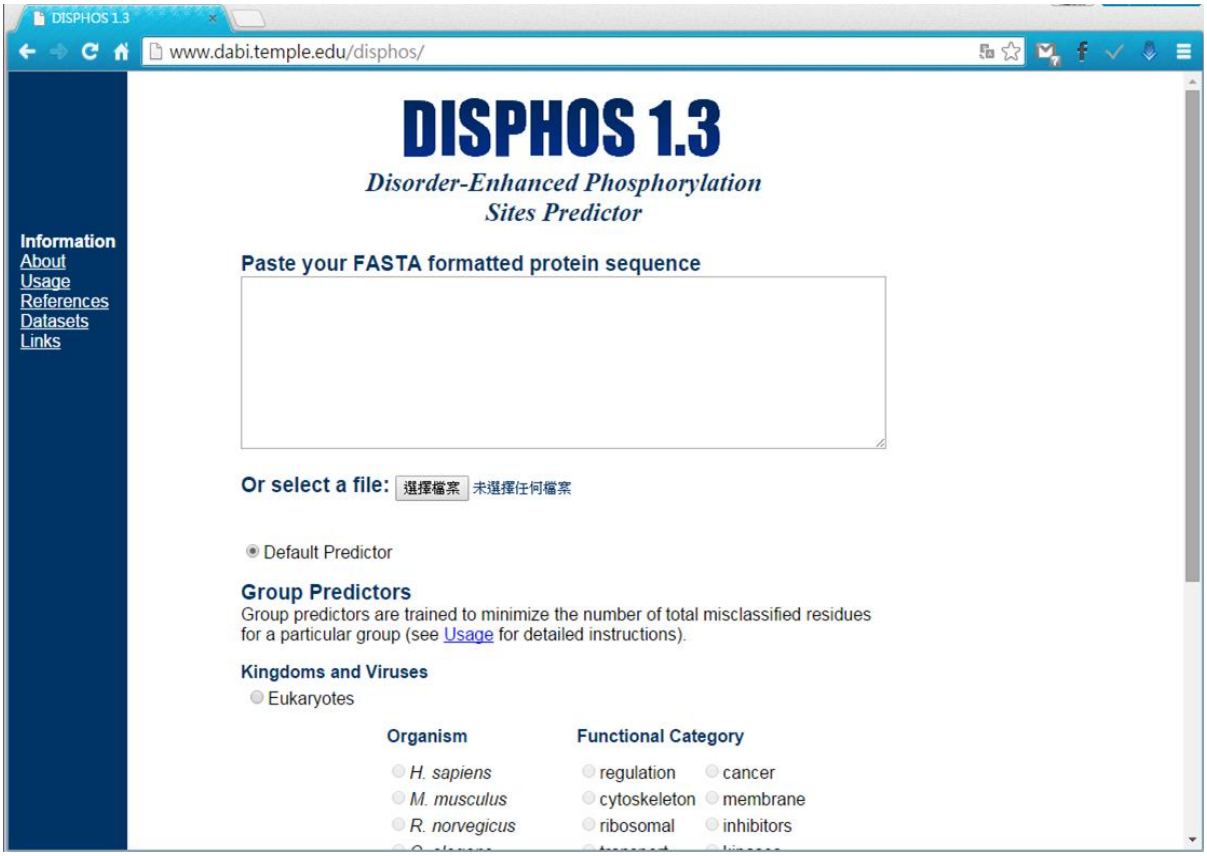

Figure 5. Home page of DISPHOS1.3

Phylogeny

Phylogenetic analysis of Cu/Zn SOD obtained from the 14 multicellular and 1 unicellular organisms were performed using multiple sequence alignments. Multiple sequence alignments were performed using Clustal Omega. It is a general purpose multiple sequence alignment program for proteins that attempts to calculate the preeminent match for the selected sequences, and lines them up so that the identities, similarities and differences can be seen. First, the protein sequences in FASTA format was submitted to Clustal Omega server, accurate alignment method was used to perform the pairwise alignments to generate the guide tree.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

Several phosphorylation sites were detected in the Cu/Zn SOD indicate the fact that it may also be regulated by the mechanism of protein phosphorylation. The three most abundant residues that are most frequently phosphorylated were serine (S), threonine (T)and tyrosine (Y). From the taken animals I get the following data. These are given in following tables.

The data obtained from the study are presented in tabular form as follows. The total serine in *H. sapiens*, *M. musculus*, *M. undulates*, *T. elegans*, *C. carpio*, *C. elegans*, *S. cerevisiae* are 10, 10, 14, 11, 9, 28, 11 but their phosphorylated sites are 2, 1, 2, 2, 1, 6, and 3 respectively. There are several organisms which have no phosphorylated serine present like *R. norvegicus*, *G. gallus*, *X. laevis*, *D. rerio* etc. (Table 2).

Table 2. Presence of phosphorylated serine(S) in taken animals

Organisms	**Systemic position**	**Total Serine (S)**	**Phosphory-lated Serine (S)**	**Percentage of Phosphorylation**	**Wet lab validation**
Homo sapiens	Kingdom: Animalia Phylum: Chordata Class: Mammalia Order: Primates Family: Hominidae Genus: *Homo* Species: *sapiens*	10	2	20.00	2
Rattus norvegicus	Kingdom: Animalia Phylum: Chordata Class: Mammalia Order: Rodentia Family: Muridae Genus: *Rattus* Species: *norvegicus*	9	0	0.00	0
Mus musculus	Kingdom Animalia Phylum: Chordata Class: Mammalia Order: Rodentia Family:Muridae Genus: *Mus* Species: *musculus*	10	1	10.00	1
Gallus gallus	Kingdom: Animalia Phylum: Chordata Class: Aves Order: Galliformes Family:Phasianidae Genus: *Gallus* Species: *gallus*	7	0	0.00	0
Melopsittacus undulatus	Kingdom: Animalia Phylum: chordate Class: Aves Order: Psittaciformes Family: Psittaculidae Genus: *Melopsittacus* Species: *undulatus*	14	2	14.266	2

Thamnophis elegans	Kingdom: Animalia Phylum: Chordata Class: Reptilia Order: Squamata Family: Colubridae Genus: *Thamnophis* Species: *elegans*	11	2	18.182	2
Xenopus laevis	Kingdom: Animalia Phylum: Chordata Class: Amphibia Order: Anura Family:Pipidae Genus: *Xenopus* Species*: laevis*	9	0	0.00	0
Danio rerio	Kingdom: Animalia Phylum: Chordata Class: Actinopterygii Order: Cypriniformes Family: Cyprinidae Genus: *Danio* Species: *rerio*	6	0	0.00	0
Cyprinus carpio	Kingdom: Animalia Phylum: Chordata Class: Actinopterygii Order: Cypriniformes Family: Cyprinidae Genus: *Cyprinus* Species: *carpio*	9	1	11.111	1
Apostichopus japonicus	Kingdom: Animalia Phylum: Echinodermata Class: Holothuroidea Order: Aspidochirotida Family: Stichopodidae Gens: *Apostichopus* Species: *japonicus*	0	0	0.00	0
Mytilus edulis	Kingdom:Animalia Phylum:Mollusca Class:Bivalvia Order:Mytiloida Family:Mytilidae Genus: *Mytilus* Species:*edulis*	8	0	0.00	0
Drosophila melanogaster	Kingdom: Animalia Phylum: Arthropoda Class:Insecta Order:Diptera Family:Drosophilidae Genus:*Drosophila* Species:*melanogaster*	9	0	0.00	0
Bombus ignitus	Kingdom: Animalia Phylum: Arthropoda Class: Insecta Order: Hymenoptera Family: Apidae Genus: *Bombus* Species: *ignitus*	6	0	0.00	0
Caenorhabditis elegans	Kingdom: Animalia Phylum: Nematoda Class: Chromadorea Order: Rhabditida Family: Rhabditidae Genus:*Caenorhabditis* Species: *elegans*	28	6	21.429	6
Saccharomyces cerevisiae	Kingdom: Fungi Phylum: Ascomycota Class: Saccharomycetes Order: Saccharomycetales Family: Saccharomycetaceae Genus: *Saccharomyces* Species: *cerevisiae*	11	3	27.273	3

The total threonine present in *G. gallus*, *T. elegans*, *X. laevis*, *C. carpio*, *A. japonicas*, *M. edulis*, *D. melanogaster*, *C, elegans*, *S. cerevisiae* are 9, 7, 7, 11, 15, 15, 9, 15, 10 but their phosphorylated sites are 1, 2, 1, 1, 1, 1, 1, 1 and 3 respectively but rest animals do not have phosphorylated threonine sites (Table 3).

Table 3. Presence of phosphorylated Threonine (T) in taken animals

Organisms	**Systemic position**	**Total Threonine (T)**	**Phosphory-lated Threonine(T)**	**Percentage of Phosphorylation**	**Wet lab validation**
Homo sapiens	Kingdom: Animalia Phylum: Chordata Class: Mammalia Order: Primates Family: Hominidae Genus: *Homo* Species: *sapiens*	8	0	0.00	0
Rattus norvegicus	Kingdom: Animalia Phylum: Chordata Class: Mammalia Order: Rodentia Family: Muridae Genus: *Rattus* Species: *norvegicus*	9	0	0.00	0
Mus musculus	Kingdom Animalia Phylum: Chordata Class: Mammalia Order: Rodentia Family:Muridae Genus: *Mus* Species: *musculus*	9	0	0.00	0
Gallus gallus	Kingdom: Animalia Phylum: Chordata Class: Aves Order: Galliformes Family:Phasianidae Genus: *Gallus* Species: *gallus*	9	1	11.111	1
Melopsittacus undulatus	Kingdom: Animalia Phylum: chordate Class: Aves Order: Psittaciformes Family: Psittaculidae Genus: *Melopsittacus* Species: *undulatus*	8	0	0.00	0
Thamnophis elegans	Kingdom: Animalia Phylum: Chordata Class: Reptilia Order: Squamata Family: Colubridae Genus: *Thamnophis* Species: *elegans*	7	2	28.571	2
Xenopus laevis	Kingdom: Animalia Phylum: Chordata Class: Amphibia Order: Anura Family:Pipidae Genus: *Xenopus* Species*: laevis*	7	1	14.286	1

Danio rerio	Kingdom: Animalia Phylum: Chordata Class: Actinopterygii Order: Cypriniformes Family: Cyprinidae Genus: *Danio* Species: *rerio*	14	0	0.00	0
Cyprinus carpio	Kingdom: Animalia Phylum: Chordata Class: Actinopterygii Order: Cypriniformes Family: Cyprinidae Genus: *Cyprinus* Species: *carpio*	11	1	9.091	1
Apostichopus japonicus	Kingdom: Animalia Phylum: Echinodermata Class: Holothuroidea Order: Aspidochirotida Family: Stichopodidae Gens: *Apostichopus* Species: *japonicus*	15	1	6.667	1
Mytilus edulis	Kingdom:Animalia Phylum:Mollusca Class:Bivalvia Order:Mytiloida Family:Mytilidae Genus: *Mytilus* Species:*edulis*	15	1	6.667	1
Drosophila melanogaster	Kingdom: Animalia Phylum: Arthropoda Class:Insecta Order:Diptera Family:Drosophilidae Genus:*Drosophila* Species:*melanogaster*	9	1	11.111	1
Bombus ignitus	Kingdom: Animalia Phylum: Arthropoda Class: Insecta Order: Hymenoptera Family: Apidae Genus: *Bombus* Species: *ignitus*	9	0	0.00	0
Caenorhabditis elegans	Kingdom: Animalia Phylum: Nematoda Class: Chromadorea Order: Rhabditida Family: Rhabditidae Genus:*Caenorhabditis* Species: *elegans*	15	1	6.667	1
Saccharomyces cerevisiae	Kingdom: Fungi Phylum: Ascomycota Class: Saccharomycetes Order: Saccharomycetales Family: Saccharomycetaceae Genus: *Saccharomyces* Species: *cerevisiae*	10	3	30.00	3

Similarly the total number of tyrosine present in *R. norvegicus*, *M. musculus*, *M. undulatus*, *B. ignites*, *S. cerevisiae* are 1, 10, 1, 1, 1, 1, 1 and their phosphorylated sites are 1, 2, 1, 1, 1, 1, 1 and there are some observed animals which have zero phosphorylated tyrosine present in there protein (Table 4).

Table 4: Presence of phosphorylated Tyrosine (Y) in taken animals

Organisms	**Systemic position**	**Total Tyrosine (Y)**	**Phosphory-lated Tyrosine (Y)**	**Percentage of Phosphorylation**	**Wet lab validation**
Homo sapiens	Kingdom: Animalia Phylum: Chordata Class: Mammalia Order: Primates Family: Hominidae Genus: *Homo* Species: *sapiens*	0	0	0.00	0
Rattus norvegicus	Kingdom: Animalia Phylum: Chordata Class: Mammalia Order: Rodentia Family: Muridae Genus: *Rattus* Species: *norvegicus*	1	0	0.00	0
Mus musculus	Kingdom Animalia Phylum: Chordata Class: Mammalia Order: Rodentia Family:Muridae Genus: *Mus* Species: *musculus*	1	1	100	1
Gallus gallus	Kingdom: Animalia Phylum: Chordata Class: Aves Order: Galliformes Family:Phasianidae Genus: *Gallus* Species: *gallus*	0	0	0.00	0
Melopsittacus undulatus	Kingdom: Animalia Phylum: chordate Class: Aves Order: Psittaciformes Family: Psittaculidae Genus: *Melopsittacus* Species: *undulatus*	10	2	20.000	2
Thamnophis elegans	Kingdom: Animalia Phylum: Chordata Class: Reptilia Order: Squamata Family: Colubridae Genus: *Thamnophis* Species: *elegans*	1	0	0.00	0
Xenopus laevis	Kingdom: Animalia Phylum: Chordata Class: Amphibia Order: Anura Family:Pipidae Genus: *Xenopus* Species*: laevis*	1	0	0.00	0
Danio rerio	Kingdom: Animalia Phylum: Chordata Class: Actinopterygii Order: Cypriniformes Family: Cyprinidae Genus: *Danio* Species: *rerio*	1	1	100	1
Cyprinus carpio	Kingdom: Animalia Phylum: Chordata Class: Actinopterygii Order: Cypriniformes Family: Cyprinidae Genus: *Cyprinus* Species: *carpio*	1	1	100	1

Apostichopus japonicus	Kingdom: Animalia Phylum: Echinodermata Class: Holothuroidea Order: Aspidochirotida Family: Stichopodidae Gens: *Apostichopus* Species: *japonicus*	0	0	0.00	0
Mytilus edulis	Kingdom:Animalia Phylum:Mollusca Class:Bivalvia Order:Mytiloida Family:Mytilidae Genus: *Mytilus* Species:*edulis*	0	0	0.00	0
Drosophila melanogaster	Kingdom: Animalia Phylum: Arthropoda Class:Insecta Order:Diptera Family:Drosophilidae Genus:*Drosophila* Species:*melanogaster*	1	1	100	1
Bombus ignitus	Kingdom: Animalia Phylum: Arthropoda Class: Insecta Order: Hymenoptera Family: Apidae Genus: *Bombus* Species: *ignitus*	1	1	100	1
Caenorhabditis elegans	Kingdom: Animalia Phylum: Nematoda Class: Chromadorea Order: Rhabditida Family: Rhabditidae Genus:*Caenorhabditis* Species: *elegans*	8	0	0.00	0
Saccharomyces cerevisiae	Kingdom: Fungi Phylum: Ascomycota Class: Saccharomycetes Order: Saccharomycetales Family: Saccharomycetaceae Genus: *Saccharomyces* Species: *cerevisiae*	1	1	100	1

Serine phosphorylation sites are found to be highest in *Homo sapiens*, *Melopsittacus undulates* and *Thamnophis elegans* that is 2 and lowest in rest of other animals except *Musmusculus* and *Cyprinus carpio.* Phosphorylation sites at the threonine residue were highest in *Thamnophis elegans* that is 2 and lowest in class mammalia that is 0.The tyrosine residue was highest in *Melopsittacus undulates* that is 2 (Fig. 6).

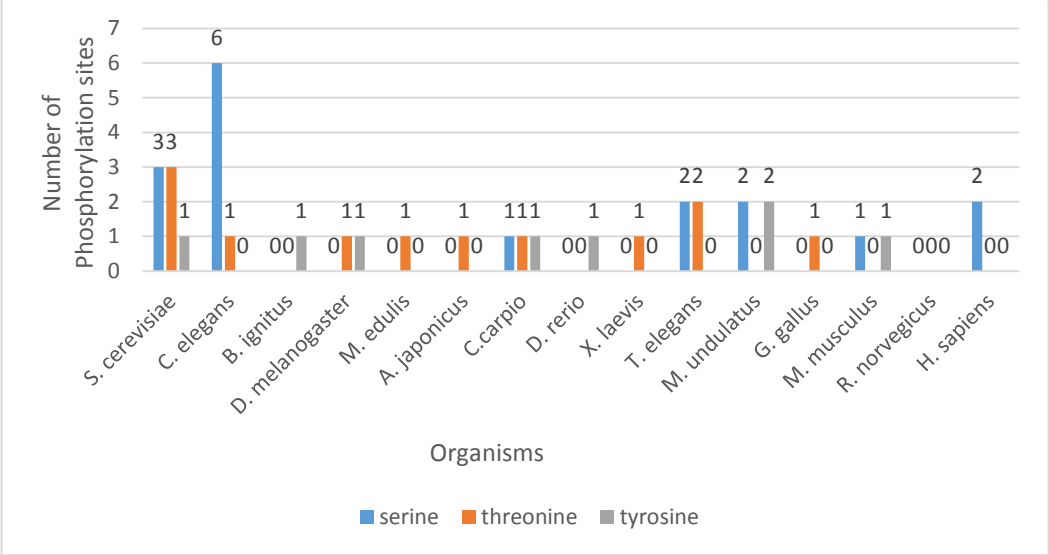

Figure 6. Distribution of phosphorylation sites (STY) in the Cu/Zn SOD in different eukaryotic species. Most abundant phosphorylation site in the Cu/Zn SOD subunit was at the serine residue with 17 phosphorylation sites. Phosphorylation sites at threonine and tyrosine residues were found to be 12 and 8 respectively in all the 15 species.

The program generated by the multiple sequence alignment (EBI, Clustal Omega) showed that the Cu/Zn SOD in *Homo sapiens*, *Rattus norvegicus* and *Mus musculus*, *Gallus gallus* and *Thamnophis elegans* were originated from the same node (Fig. 7).

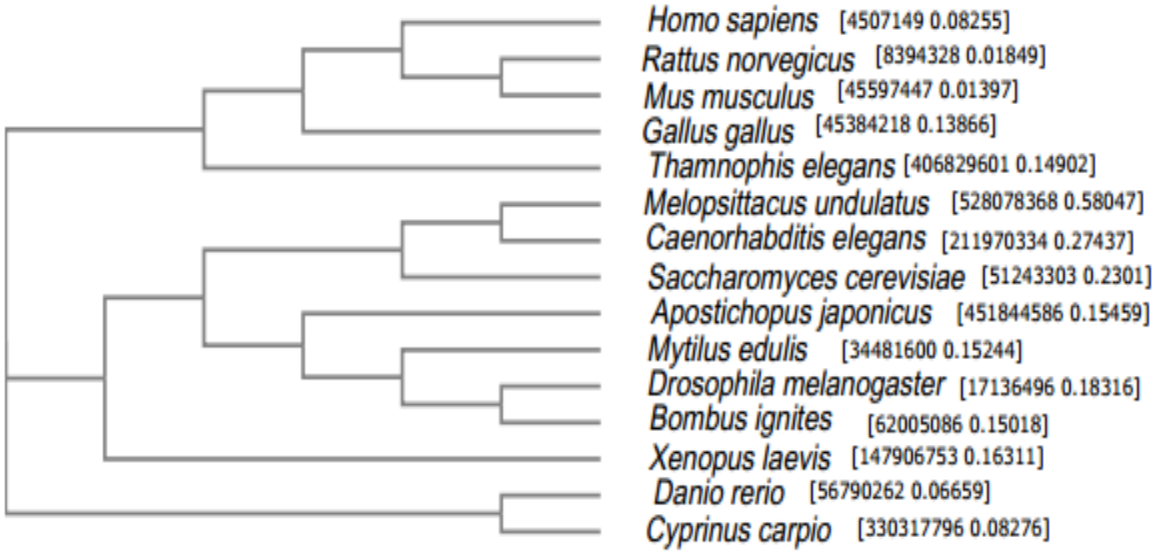

Figure 7. Phylogenetic tree depicting evolutionary relationships of Cu/Zn SOD subunits between different organisms.

Protein phosphorylation regulates the function of proteins by phosphor-regulation which is the most important determinants of signalling systems. It is an extremely complex procedure on a proteome-wide scale due to a great number of modifying proteins that are modified by these enzymes, and the variant nature of protein expression during different cellular programs. The critical biological processes are regulated by many of these phosphorylation sites and may provide evidence for diagnostic or therapeutic targets for molecular medicine. Hence, the identification and detection of phosphorylation sites on a wide diversity of cellular proteins are remarkably essential to understand the signalling proteins and pathways concerned in disease state progression. In the last two decades, researchers have developed several tools for the experimental and computational identification of sequence and structural motifs which are responsible for encoding the kinase-substrate interaction residues and the phosphorylated amino acid itself.

Protein phosphorylation is a reversible posttranslational modification that can regulate the role of protein in several physiological processes in almost every possible way. Protein phosphorylation is the covalent binding of a phosphate group to some critical amino acid residues of the protein by the enzyme protein kinase. Protein kinase transfers the gamma phosphate group from ATP/GTP to hydroxyl groups of specific amino acid side chains like serine, threonine and tyrosine residues. Another enzyme protein phosphate catalyses the reverse reaction of phosphorylation and removes the phosphate group from protein. The phosphorylation of specific residues induces structural changes that regulate protein functions by modulating protein folding, substrate affinity, stability and activity. Phosphorylation acts as a molecular switch for many regulatory events in signalling pathways that drive cell division, proliferation, differentiation and apoptosis. Thus phosphorylation act as a major regulator of protein activity in every aspect of cellular processes. So the study of protein phosphorylation provides information about the activity and regulation of protein in various activities within cell.

Superoxide is one of the main reactive oxygen species in the cell. As a consequence, SOD serves a key antioxidant role. The physiological importance of SODs is illustrated by lacking SOD2 in mice causes' death several days after birth, amid massive oxidative stress. Mice lacking SOD1 develop a wide range of pathologies, including hepatocellular carcinoma, an acceleration of age-related muscle mass loss, an earlier incidence of cataracts and a reduced lifespan. Mice lacking SOD3 do not show any obvious defects and exhibit a normal lifespan, though they are more sensitive to hyperoxic injury. Mice of any SOD enzyme are more sensitive to the lethal effects of superoxide-generating drugs, such as paraquat and diquat.

Drosophila lacking SOD1 have a dramatically shortened lifespan, whereas flies lacking SOD2 die before birth. SOD knockdowns in *C. elegans* do not cause major physiological disruptions. Knockout or null mutations in SOD1 are highly detrimental to aerobic growth in the yeast *Saccharomyces cerevisiae* and result in a dramatic reduction in post-diauxic lifespan.Several prokaryotic SOD null mutants have been generated, including *E. coli*. The loss of periplasmic Cu/Zn SOD causes loss of virulence and might be an attractive target for new antibiotics. Mutations in the first SOD enzyme (SOD1) can cause familial amyotrophic lateral sclerosis (ALS, a form of motor neuron disease).

The study of Cu/Zn SOD and its regulation by phosphorylation is well studied in case of vertebrates. Various researches have been done on several vertebrates for examining the role of SOD in regulation of oxidative stress. In case of invertebrates various experiments have been done to illustrate the role of SOD in phylums like Echinodermata, Mollusca, Arthropoda, Annelida and Nematoda. But in case of phylum Platyhelminthes the study of superoxide is highly limited. Mostly partial studies on SOD have been found in case of phylum Porifera and Coelenterata. So further studies on SOD in these phylums can provide new dimensions in the regulation of oxidative stress and many experiments conducted on Archea revealed the presence of Cu/Zn SOD, which protect them from reactive oxygen species.

CONCLUSION

CHAPTER-V

CONCLUSION

Protein phosphorylation described as an important transcriptional regulation that highly modify and regulate the protein activity in various cellular functions like signal transduction, growth, metabolism, division, differentiation, apoptosis etc. Protein kinase acts as the major protein phosphorylating enzyme which acts by transferring gamma phosphate group from ATP or GTP to its protein substrate. Phosphorylation induce conformational changes in protein structure as a result of which the protein either become activated or deactivated. An enzyme called phosphatase which dephosphorylate the protein so that protein may be deactivated.

The superoxide dismutase enzyme is found in almost every living organisms. It protects cells from oxidative stress. This study on Cu/Zn SOD in 14 multicellular organisms and 1 unicellular organism emphasizes the phosphorylation sites in them. These phosphorylation sites were analysed by DISPHOS scores. These scores obtained from DISPHOS results are helpful in predicting the phosphorylation sites of Cu/Zn SOD and its regulation in organisms using wet laboratory approaches.

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