Mutational analysis of PDC-109 and effect of polyamines and molecular crowding on its functional activities

A thesis
Submitted for the degree of
DOCTOR OF PHILOSOPHY

By

Bhanu Pratap Singh

School of Chemistry
University of Hyderabad
Hyderabad – 500 046
INDIA
December, 2015

Contents

School of Chemistry
University of Hyderabad
Hyderabad, 500046

STATMENT

I hereby declare that the matter embodied in this thesis is the result of investigation carried out by me in the School of Chemistry, University of Hyderabad, Hyderabad, under the supervision of **Prof. Musti J. Swamy**.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made whenever the work described is based on the finding of other investigators. Any omission which might have occurred by oversight or error is regretted.

Hyderabad **Bhanu Pratap Singh**

December, 2015

School of Chemistry
University of Hyderabad
Hyderabad, 500046

CERTIFICATE

Certified that the work embodied in this thesis entitled **“Mutational analysis of PDC-109 and effect of polyamines and molecular crowding on its functional activities”** has been carried out by Mr. Bhanu Pratap Singh under my supervision and the same has not been submitted elsewhere for any degree.

Hyderabad
December, 2015

Prof. Musti J. Swamy
(Thesis Supervisor)

Dean
School of Chemistry

Acknowledgment

First and foremost I wish to acknowledge Prof. Musti J. Swamy, my Ph.D. supervisor for providing excellent guidance and all the resources to carry out the present work. He helped me a lot with his enriched scientific experience do design the experiments with proper planning and in developing the skill for making new ideas. I feel the training I got under him will be very useful in my future research. I am indeed very much thankful to him.

I thank Prof. M. Durga Prasad, Dean, School of Chemistry and former Deans, Prof. M. V. Rajasekharan and D. Basavaia for providing excellent infrastructure research facility to carry out the research. I am also thankful to my doctoral committee member Prof. Abani K. Bhuyan and Prof. Lalitha Guruprsad for their kind support and encouragement. I am also thankful to non teaching staff of the department and university administration for providing me valuable service at various times. I thank central instrument facility of the University for providing me the facility for confocal microscopic and circular dichroism studies. I am also very much thankful to Dr. Vinu and Dr. Dharmarao from Lam Form, Guntur. Who generously provided the bovine seminal plasma for carrying out my present study.

I am also greatly thankful to Dr. Ch. Mohan Rao (Director CCMB, Hyderabad) for allowing me to work in his lab for molecular biology experiments. During my working period at CCMB, Dr. T. Ramakrishna Murthy (Chief Scientist, CCMB) helped me a lot to complete the experiments successfully. I am very much thankful to him.

At the time of joining of lab, Rajeshwer introduce me with various biophysical techniques, which were useful throughout my Ph.D. period. He also provided me bacterial clone of PDC-109, which was very useful for carry out the one of important part of present study. During my work at CCMB Abhishek introduced me with various molecular biology techniques and helped me to get access to all the required instrumental facility. I am very much thankful to both of them.

I would like to acknowledge all the lab members for making a good working environment and their co-operation during entire Ph.D. time. Useful discussion and support from Kishore, Thirupathi, Pavan, Siva, Sudhir and Debparna helped me to solve various experimental problems and widening of my knowledge on the subject. Working with all of them is a great time for me. I also want to acknowledge Amrita, who worked with me for her M.Sc. project. I appreciate her significant contribution to purify the mutant proteins, which was extremely useful for the study presented in Chapter 2. I also want to acknowledge Dr. Isita Saha, who joined the lab as a post doctoral fellow and I appreciate her contribution to carry out the work presented in Chapter 3 and 4. It was great to find many good friends like Satish, Narayan, Sanjeev, Balaswamy, Sashi, Yasin, Ramakrishna, Sunil, Chandrashekhar, Keshav in the department and Vani, Prashant, Suvarsha, Saad, Kranti, Madhuri, Abdulla, Ann, Praveena and Gopi at CCMB who made the Ph.D. time memorable and enjoyable. During my Ph.D. period many project students and research scholar from different institutes visited our lab for short periods at different times. Sneha, Supreeth, Sonali, Sandeep, Gayatri, Kejiya, Tejashwi, Bianca, Pabulo, Madhuprakash, Siri, Sritama, Saket, Minu, Harishankar, Deepjyoti, and many others. I appreciate all of them for enriching my experience in the lab.

I got a lot of encouragement from my friends without their support it was not possible to complete this work. My friends Prachi, Vivek, Reejuana, Sony, Satya, Mohit, Sai, Kapil Sharma, Kapil Manglani, Saurabh, Mahesh, Mastan, Ajay, Debobrat, Jeetendra always encouraged me and shown their support all the time. I highly acknowledge to all of them.

I am very much thankful to DST for providing the funding to carry out the research and to UGC for providing the fellowship for entire period of Ph.D.

I acknowledge my parents for providing me freedom and invaluable support. My mother had always supported me and appreciated my work and has always shown the great patience which is very much important to carry out the research work. I have a feeling of special gratitude for Rashmi for finding in her a wonderful companion, present work would not have taken the shape without her support. Finally, I am thankful to Almighty God for providing me the inner guidance.

Bhanu Pratap Singh

Abbreviation

ADH - Alcohol dehydrogenase

ADP - Adenosine di phosphate

Ala (A) - Alanine

AFM - Atomic force microscopy

AR - Acrosome reaction

ATP - Adenosine tri phosphate

bis-ANS - 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulphonic acid

BSP - Bovine seminal plasma

CA - Carbonic anhydrase

CD - Circular dichroism

CLA - Chaperone like activity

Da - Dalton

DTT - Dithiothreitol

DSC - Differential scanning calorimetry

DMPC - 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine

DEAE - Diethylaminoethyl

EDTA - Ethylenediamine tetra acetic acid

FnII - Fibronectin type II

FP - Forward primer

G6PD - Glucose-6-phosphate dehydrogenase

GdnHCl - Guanidine hydrochloride

GSH - Glutathione reduced

GSSG - Glutathione oxidised

HCl - Hydrochloric acid

HPLC - High performance liquid chromatography

IPTG - Isopropyl β-D-1-thiogalactopyranoside

ITC - Isothermal titration calorimetry

K_a - Association constant

LDH - Lactate Dehydrogenase

M - Methionine

MRE - Mean Residual Ellipticity

NaCl - Sodium Chloride

NADP - Nicotinamide adenine dinucleotide phosphate

NAD - Nicotinamide adenine dinucleotide

NMR	-	Nuclear magnetic resonance
OD	-	Optical Density
PAGE	-	Poly Acrylamide Gel Electrophoresis
PC	-	Phosphatidylcholine
PCR	-	Polymerase chain reaction
PDB	-	Protein data bank
PDC-109	-	BSP-A1/-A2
PrC	-	Phosphoryl Choline
RBC	-	Red blood cells
rPDC-109	-	Recombinant PDC-109
RP	-	Reverse Primer
SEM	-	Scanning Electron Microscope
SUVs	-	Small Unilamellar Vesicles
SP	-	Seminal plasma
Spm	-	Spermine
Spd	-	Spermidine
SDS	-	Sodium dodecyl sulphate
SDM	-	Site directed mutagenesis

SPR Surface plasmon resonance

TBS I - Tris buffer containing, 50 mM Tris, 0.15 M NaCl, 5mM EDTA, 0.025% sodium azide, pH = 7.4

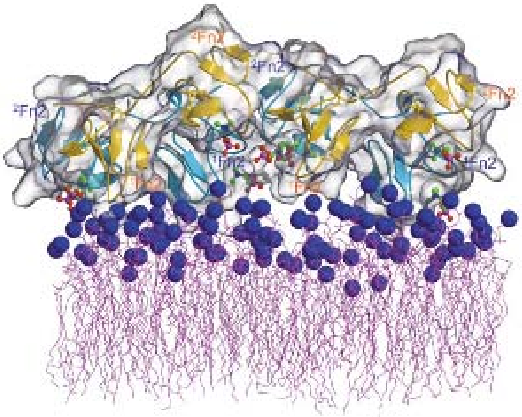
TBS II - Tris buffer containing, 25 mM Tris, 1 M NaCl, 0.025% NaN3, pH = 6.4

Trp(W) - Tryptophan

ZP - Zona pellucida

Chapter 1

Introduction



Binding of lipid head group to fibronectin type II domain of PDC-109. Reproduced from *Structure*, Vol. 10, 505–514.

1. Reproduction

Reproduction is a fundamental feature of all forms of life, by which new organisms are produced from their parents. There are various mode of reproduction, which are categorized under two forms: sexual and asexual.

1.1 Asexual reproduction

It is a primitive mode of reproduction, which requires only a single parent. In asexual reproduction new offspring contains same genetic material as parent organism. Single-celled organisms such as the archaebacteria, eubacteria, protists and as well as many plants and fungi reproduce asexually. Examples of asexual reproduction are: fission, fragmentation, budding, spore formation and vegetative reproduction.

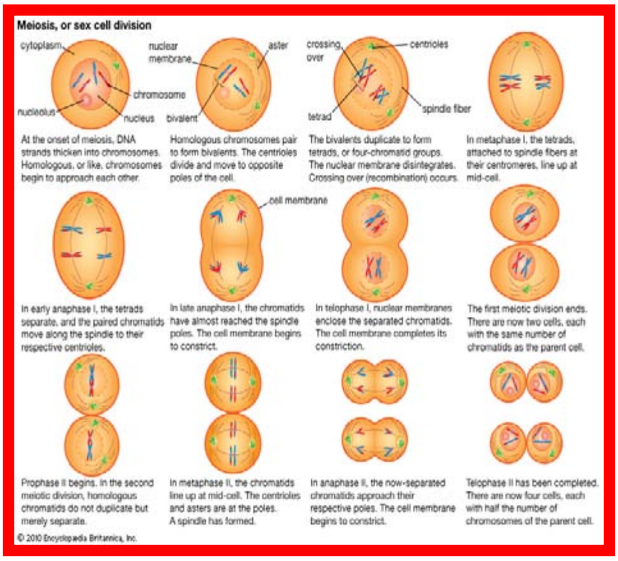
1.2 Sexual reproduction

It involves genetic material from two organisms. Most of the higher organisms reproduce by sexual reproduction. Process of sexual reproduction starts with the formation of gametic cell by meiosis, in which the resultant cell contains half of the genetic material of each parent cell (Figure 1). At the time of fertilization gametic cells from two different parents combine and the amount of genetic material is restored in the offspring.

2. Fertilization

Fertilization is a process by which nucleus of the male gametic cell (or spermatozoa) unites with the nucleus of the female gametic cell (or ovum) to form a zygote. Fusion of nuclei of two gametic cells results in the restoration of genetic content as each gametic cell contains a single copy of chromosome typical of the species.

The first significant event in fertilization is fusion of plasma membrane of two gametic cells. Many factors present on the surface of plasma membrane of both gametic cells play important roles in this process and subsequent disruption of plasma membrane at the point of fusion. It results in the formation of a channel, through which nucleus of the male gametic cell enters the female gametic cell and fuses with its nucleus and the process of fertilization completes (Figure 2). The most important result of fertilization is activation of metaphase II arrested ovum cell. Once ovum cell is activated it rapidly divides through mitotic division, which is important for embryo formation.



At the onset of meiosis, DNA strands thicken into chromosomes. Homologous, or like, chromosomes begin to approach each other.

Homologous chromosomes pair to form bivalents. The centrioles divide and move to opposite poles of the cell.

The bivalents duplicate to form tetrads, or four-chromatid groups. The nuclear membrane disintegrates. Crossing over (recombination) occurs.

In metaphase I, the tetrads, attached to spindle fibers at their centromeres, line up at mid-cell.

In early anaphase I, the tetrads separate, and the paired chromatids move along the spindle to their respective centrioles.

In late anaphase I, the chromatids have almost reached the spindle poles. The cell membrane begins to constrict.

In telophase I, nuclear membranes enclose the separated chromatids. The cell membrane completes its constriction.

The first meiotic division ends. There are now two cells, each with the same number of chromatids as the parent cell.

Prophase II begins. In the second meiotic division, homologous chromatids do not duplicate but merely separate.

In metaphase II, the chromatids line up at mid-cell. The centrioles and asters are at the poles. A spindle has formed.

In anaphase II, the now-separated chromatids approach their respective poles. The cell membrane begins to constrict.

Telophase II has been completed. There are now four cells, each with half the number of chromosomes of the parent cell.

Figure 1. Meiosis or reduction devision. A germ cell first dividing into two cells, each cell carrying one set of homologos chromosomes. In the second meiotic divison sister chromatids from each chromosome segregate and make two new cells. Thus from one germ cell, four gamete are formed, each carrying half of the genetic material of the parent cell. Reproduced from: http://www.britannica.com/

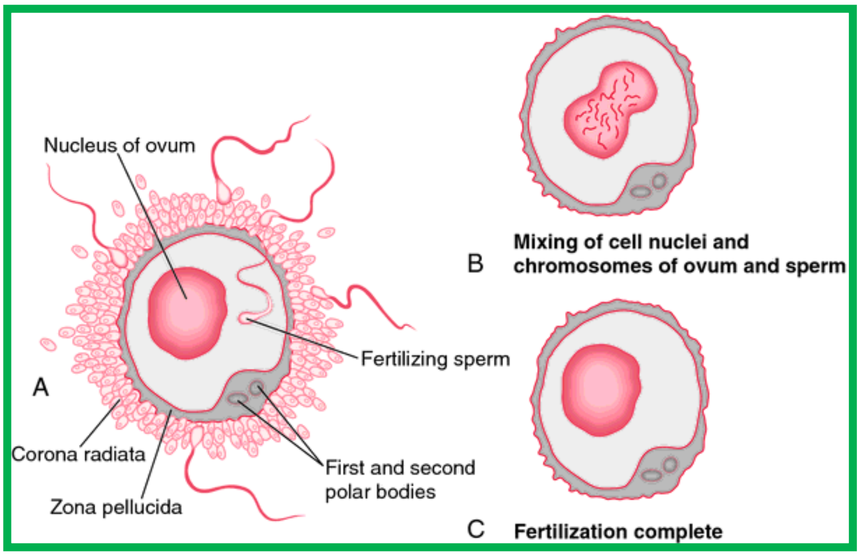


Figure 2. Fertilization and zygote formation. (A) A sperm enters the ovum. **(B)** Haploid chromosome of the sperm mingles with the haploid chromosome of ovum and restores the diploid number of chromosome. **(C)** The fertilized ovum, now called a zygote, is ready for first mitotic cell division. Reproduced from: http://medical-dictionary.thefreedictionary.com/

3. Sperm: The male gamete

Sperm is the male reproductive cell. In higher vertebrates, especially in mammals, sperm cells are produced in testes by stem cells, which are arranged in the basal lamina in seminiferous tubuli (Flesch et al., 2000). These stem cells (type A spermatogonia) divide several times and produce spermatocytes. Subsequently these spermatocytes divide by meiotic cell division and produce spermatides. These spermatide cell migrate toward lumen and transformed into highly differentiated and polarized cells. At the time of fertilization, nucleus of the sperm cell fuses with that of the female gametic cell (ovum) and forms the zygote, which produces a new offspring. Mature sperm cells have two distinguishable parts, a head and a tail

(Figure 3). The head portion is mainly a cell nucleus; covering the head of the sperm is a cap known as the acrosome, which contains hydrolytic enzymes that help sperm to enter an egg. A small middle portion of the sperm contains the mitochondria. The tail of the sperm, sometimes called the flagellum, is a slender, hairlike bundle of filaments that connects to the head via a middle portion, called midpiece. The tail gives the sperm cell movement, which helps it to travel to the egg to complete the event of fertilization. In most mammalian species, sperm cells are fully matured when they reach the end of the cauda of the epididymis (Eddy et al., 1994).

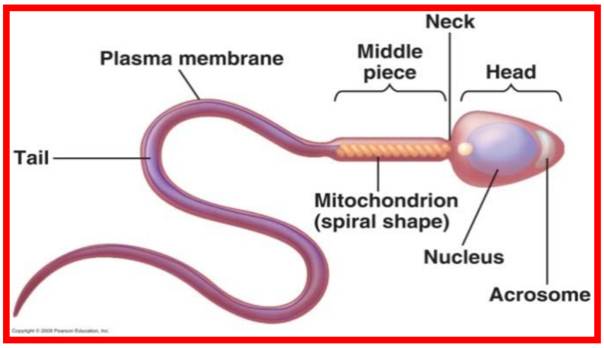


Figure 3. Sperm Cell; Epididymal mature sperm cell has characteristically three differentiated region, namely, head, middle piece and tail. Adopted from: http://www.biology.lifeeasy.org/

3.1 Sperm plasma membrane

Sperm plasma membrane plays an important role in the cross-talk between spermatozoon and oocyte, which is crucial for fertilization. Therefore study of its biochemical composition is a topic of much interest in sperm physiology and in pathological condition. During epididymal maturation spermatozoa lose most of the cell organelles which are important for active metabolism in the cell and it becomes polarized into structurally and functionally two different domains. As mature spermatozoa lack important cell organells for active metabolism which produces various component of plasma membrane i. e. lipids, proteins and cholesterol, plasma membrane of mature sperm is considered as metabolically inert. However during its transit from the epididymis, sperm plasma membrane undergoes different kind of changes by adsorbing, releasing or modification of many lipid and protein molecules on its surface. The role of these surface alterations is not fully understood, but it has been shown that many molecules bind on the sperm surface which plays an important role in fertilization, for example, in recognition of various factors present on female reproductive tract and surface of ovum cell, which leads to many structural and biochemical changes that are important prior to fertilization (Flesch et al., 2000).

3.2 Lipid composition of sperm plasma membrane

Analysis of many mammalian and non-mammalian sperm biochemical analysis showed phospholipids represent the major class of lipid in its plasma membrane with phosphatidylcholine and phosphatidylethanolamine being the major components (Mann, 1964, 1981). Although there is considerable variation between different mammalian species, in general, on the molar basis, the plasma membrane contains approximately 70% phospholipids, 25% neutral lipids and 5% glycolipids. A significant amount of phospholipids is present in the form of plasmalogens. Function of these lipids is not well known, but it is interesting to note that similar membrane lipid constituents are present in neural cells as well (Mathews and Van Holde, 1990). In this context it has been proposed that these molecules play an important role in producing intracellular second messengers by transducing the signals from outer to inner leaflet of membrane (Cornwell and Moriski, 1984).

3.3 Sperm capacitation

Freshly ejaculated mammalian sperm are unable to fertilize metaphase II arrested ovum cell. During their stay in the female reproductive tract, they interact with various factor of female reproductive tract. This leads to a series of ultrastructural and biochemical changes in the sperm, which enables the sperm to fertilize the ovum (Figure 4). This collective process of sperm maturation in female reproductive tract is termed as *sperm capacitation* (Yanagimachi, 1994). This term was first coined in 1951 by Austin and Chang based on independent studies conducted by them (Chang, 1951; Austin, 1951). Some important changes associated with sperm capacitation are the following:

1. Alteration or removal of sperm coating materials. These coating materials become adsorbed to or integrated within the sperm plasma membrane during epididymal transport and also during exposure to seminal plasma
2. A decrease in the net negative surface charge
3. Conformational changes to intrinsic membrane proteins
4. Changes in the permeability of the membrane to various ions, especially calcium
5. Increased cellular cAMP concentration and membrane fluidity.

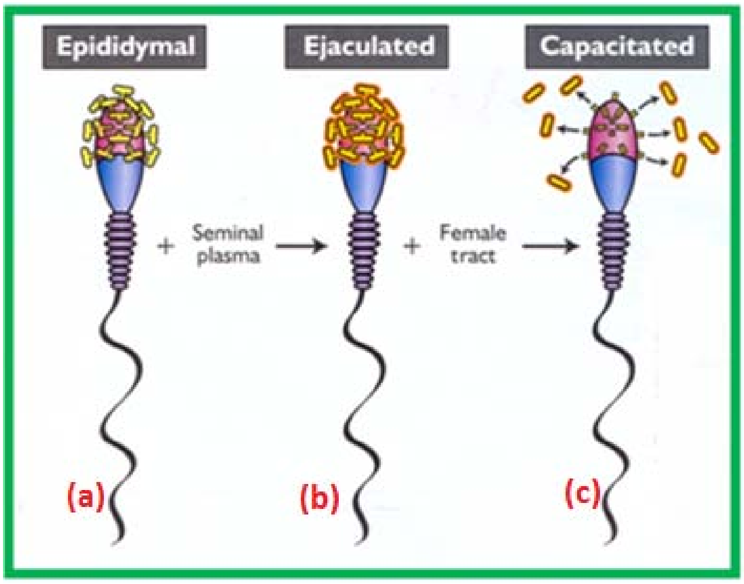


Figure 4. Sperm Capacitation. At the time of ejaculation sperm plasma membrane is associated with many proteins. In female reproductive tract these proteins interact with factors present in the environment. These interactions lead to many ultrastructural and biochemical changes in the sperm cells and make them competent to fertilize female gamete. **(a)** The plasma membrane of epididymal spermatozoa contains a complement of surface molecules (proteins and carbohydrate) illustrated here with yellow Ts. **(b)** The surface molecules in epididymal sperm become coated with seminal plasma proteins, that mask portions of the membrane. **(c)** when sperm are exposed to female tract environment, these seminal plasma coatings, along with some of the surface molecules, are removed, thus exposing portions of the molecules that can bind to the zona pellucida of the oocyte. Reproduced from: http://www.britannica.com/

4. Acrosome reaction (AR)

During the testicular development of sperm, acrosome develops from the golgi apparatus over the anterior of the sperm head. This organelle contains various hydrolytic enzymes like hyaluronidase and acrosin, which play an important role at the time of sperm—egg interaction (Toshimori, 2009). After a spermatozoon comes in contact with an egg, the acrosome undergoes a series of well-defined structural changes. At this time a structure within the acrosome, called the acrosomal vesicle, bursts, and the plasma membrane surrounding the spermatozoon fuses at the acrosomal tip with the membrane surrounding the acrosomal vesicle to form an opening. As the opening is formed, the acrosomal granule, which is enclosed within the acrosomal vesicle, disappears. Dissolution of the granule releases a substance called lysin, which breaks down the egg envelopes, allowing passage of the spermatozoon to the egg (Figure 5). This sequence of events termed as acrosomal reaction, are prerequisites for fusion of sperm nucleus with ovum cell (Yanagimachi, 1994; Brucker and Lipford, 1995). The acrosomal membrane region opposite the opening adheres to the nuclear envelope of the spermatozoon and forms a shallow outpocketing, which rapidly elongates into a thin tube, the acrosomal tubule that extends to the egg surface and fuses with the egg plasma membrane. The tubule thus formed establishes continuity between the egg and the spermatozoon and provides a way for the spermatozoal nucleus to reach the interior of the egg. Various factors have been proposed to induce the AR; however, the role of ZP3 is most accepted (Bleil and Wassarman, 1983; Ward and Kopf, 1993; Florman *et al.*, 1998). Binding of ZP3 to putative complementary receptor(s) on the sperm surface activates transmembrane signals that trigger cellular cascades resulting in the acrosome reaction (Wassarman, 1990a,b, 1999; Saling, 1991).

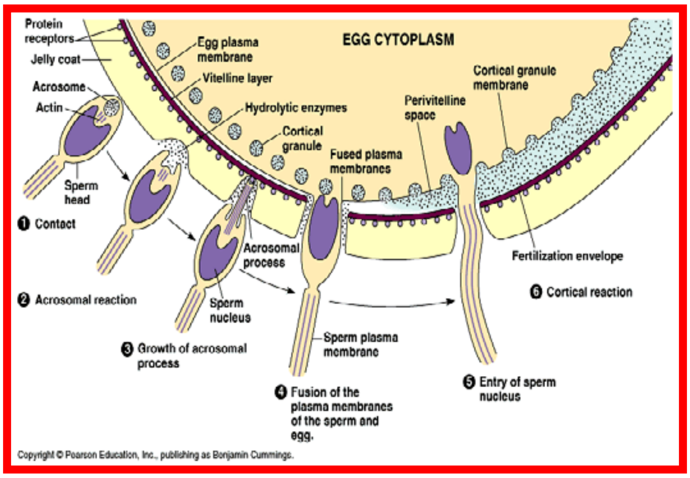


Figure 5. Acrosome reaction; Sperm-egg Membrane fusion results in acrosome reaction, which is followed by sperm nucleus transfer inside the ovum cell. Reproduced from: *Nature*, Vol. 434, 234-238.

5. Seminal plasma (SP)

The ejaculated semen consists of cellular and noncellular components. Sperm makes the cellular component. The noncellular component is called seminal plasma, which is the combined secretions, contributed by the testis, epididymis, seminal vesicles, ampullae, prostate and bulbo-urethral glands. Seminal plasma is a highly complex biological fluid (Mann, 1954). Classical view for SP is that it serves as a nutritive medium and carrier for the spermatozoa in their journey from the male testes to their target, the female uterus. However, a number of studies have shown that apart from these, proteins present in the SP play many important roles, which include modulation of sperm function, interaction between sperm plasma membrane and epithelia of female genital tract, sperm motility, sperm-egg interaction, induction of innate and adaptive immune response by the female (Martinez *et al.*, 2011; Gwathmey *et al.*, 2003). SP contains both organic and inorganic molecules of low as well as high molecular weight, viz., proteins, amino acids, enzymes, fructose and other carbohydrates, harmones, cytokines, lipids, major minerals and trace elements, ions (Na^+, K^+, Zn^+, Ca^{++}, Mg^{++}). Nitrogenous components such as polyamines, ammonia, urea, uric acid, and creatinine and reducing substances such as ascorbic acid, and hypotaurine also exist in the seminal plasma of some mammals.

5.1 Seminal plasma proteins

Analysis of SP in various mammalian species shows that proteins are the major components in it throughout species. Major fraction of proteins in the SP originate from the testis, epididymis, vas deference, prostate, seminal vesicle and bulbourethral glands. These are known as seminal plasma specific proteins. Major proteins of SP can be categorized into three main groups: proteins containing fibronectin type II (Fn-2) domain, spermadhesins and cysteine-rich secretory proteins (CRISP) (Matinez *et al.,* 2011). Substantial amount of work has been done in the last few decades which has shown that SP proteins play key roles in various important steps preceding fertilization, such as regulating sperm capacitation, formation of oviductal sperm reservoir, modulation of uterine immune response, gamete interaction and fusion (Swamy, 2004; TÖpfer-Petersen, 2005; Ignotz *et al.*, 2001).

In addition to the seminal plasma specific proteins, seminal plasma of most species contains proteins, similar to proteins in plasma of blood, viz., proalbumin, albumin, globulins, transferrin, α-antitrypsin, β-lipoprotein, β-glycoprotein, peptide hormones, immunoglobulins, complement factor, cytokines and chemokines (Huleihel *et al.,* 1996; Gutsche, 2003; Politech, 2007; Soucek, 2010). These proteins are involved in regulation of osmotic pressure and pH of seminal plasma, transport of ions, lipid and hormones. The biosynthesis and secretion of these proteins is regulated by testosterone levels in the blood.

5.2 Structure and function of the major protein of bovine seminal plasma, PDC-109

The bovine seminal plasma contains a group of four acidic proteins – designated as BSP-A1, BSP-A2, BSP-A3, and BSP-30-kDa – which bind to the spermatozoa. Collectively, these four proteins are referred to as bovine seminal plasma proteins, or as BSP proteins (Manjunath *et al.,* 1987a, b). Among all the mammalian seminal plasma proteins, these proteins have been extensively studied by different biophysical and biochemical approaches. BSP-A1 and BSP-A2 have the same primary structure, they differ only in the degree of glycosylation and their mixture is referred to as PDC-109 (Esch *et al.*, 1983 and Seidah *et al.*, 1987). PDC-109 is the major protein of bovine seminal plasma and is present at a concentration of 15–25 mg/ml in the seminal plasma, which comprises about 60% of total bovine seminal plasma proteins (Scheit *et al.*, 1988). It is a polypeptide of 109 amino acids and is composed of an N-terminal 23-residue stretch followed by two tandemly repeating fibronectin type-II (FnII) domains (Esch *et al.*, 1983; Baker *et al.*, 1985 and Seidah *et al.*, 1987). Sperm plasma membrane is very rich in phosphatidylchloline and each FnII domain of PDC-109 binds to its head group with high specificity. The three-dimensional structure of PDC-109 complexed with *O*-phosphorylcholine, solved by single-crystal x-ray diffraction reveals that the two choline-phospholipid binding sites are on the same face of the protein. Ligand binding is mediated by a cation-π interaction between the quaternary ammonium group of the choline moiety and the indole ring of a core tryptophan residue and hydrogen bonding between the phosphate group and exposed tyrosine residues of the protein. Cation-π interaction in the first FnII domain involves the core Trp residue at position 47. However, this interaction in the second FnII domain takes place with two different Trp residues. In one orientation, core Trp93 interact with ammonium group of phosphorylcholine and in second orientation instead of core Trp93, the ammonium group interacts with Trp106 (Figure 6) (Wah *et al.*, 2002).

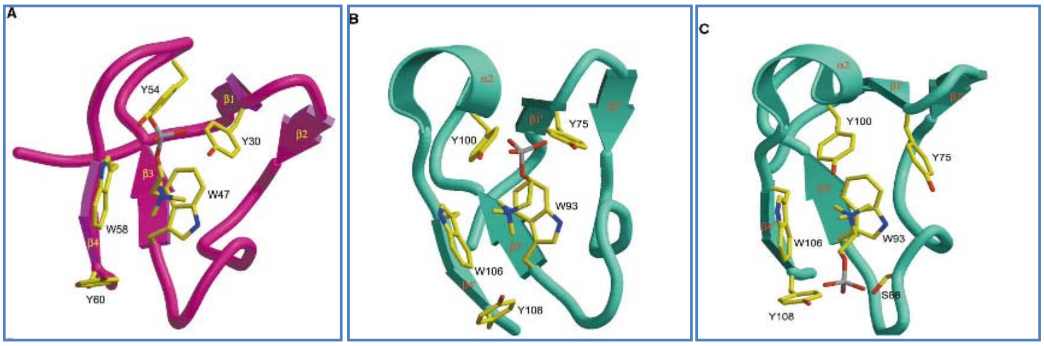


Figure 6. Structure of the FN2 domains of PDC-109. Close-up view of the relative spatial orientation and different binding conformations of phosphorylcholine molecules **(A)** 1Fn2 domains of monomers A and B. **(B)** 2Fn2 of monomer A. **(C)** 2Fn2 domain of monomer B. Reproduced from: *Structure*, Vol. 10, 505–514.

As it has been mentioned above, at the time of ejaculation PDC-109 binds to PC head group of sperm plasma membrane through the FnII domain. Binding of PDC-109 to the plasma membrane of sperm or fibroblasts results in the efflux of PC and cholesterol (referred to as cholesterol efflux). Similar studies with PC model membrane show that presence of PDC-109 destabilizes the membrane (Figure 7) (Thérien *et al.*, 1998; Damai *et al.*, 2010). These observations shed light on the importance of PDC-109 in sperm capacitation, which is a necessary event before fertilization.

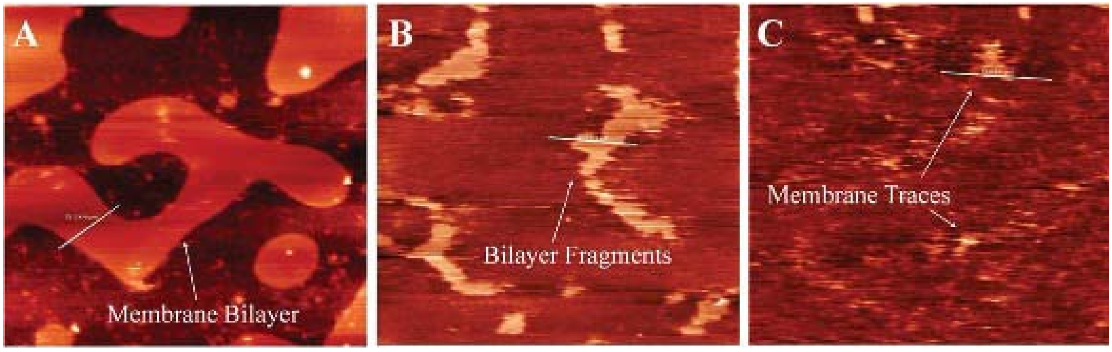


Figure 7. Atomic force microscopic studies to investigate the effect of PDC-109 on model membranes in liquid cell. **(A)** DPPC bilayer in buffer **(B)** DPPC incubated with 1.5 mg/mL of PDC-109 for 10 min **(C)** DPPC incubated with same concentration of PDC-109 for 25-30 min. All images are (3 x 3 µm) in size. Reproduced from: *Biochemistry* 49, 3908-3918.

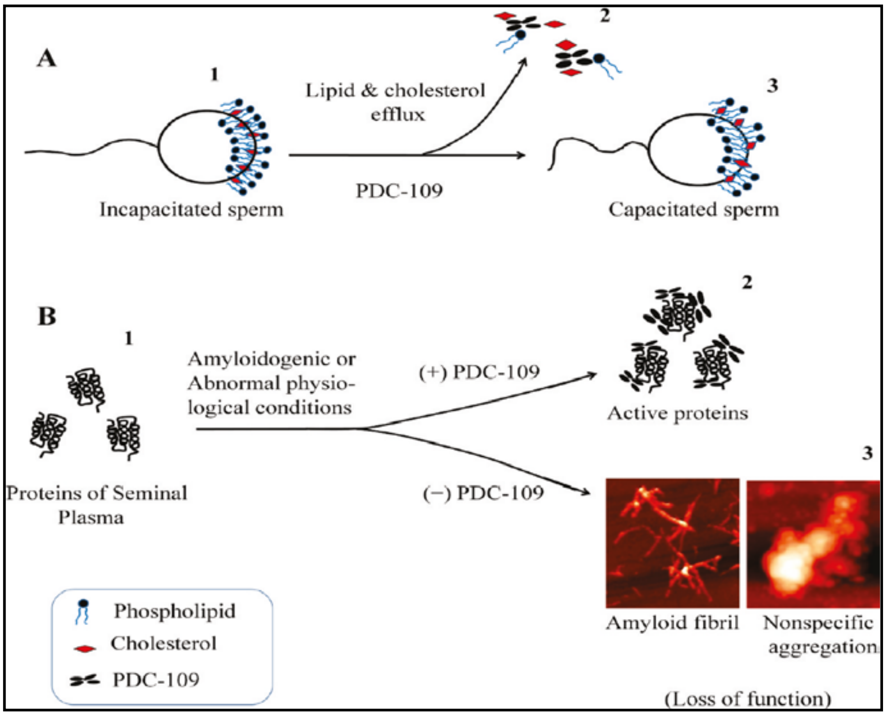


Figure 8. Schematic model for PDC-109-induced sperm capacitation and its chaperone like activity. **(A)** At the time of ejaculation PDC-109 binds to sperm plasma membrane and leads to lipid and cholesterol efflux, which is important for sperm capacitation. **(B)** Presence of PDC-109 protects target proteins from denaturing condition, in absence of PDC-109 these target protein forms amorphous and amyloid aggregates. Reproduced from: *Biochemistry* 49, 3908-3918.

Apart from binding lipid molecules on sperm plasma membrane and playing important role in sperm capacitation, PDC-109 appears to play some other important roles. In mammals, after sperm are deposited in the reproductive tract, they form oviductal sperm reservoir, where they stay before their onward journey toward the uterine tube. During this stay at oviduct many ultrastructural and biochemical changes happen, which enable the sperm to acquire the ability to fertilize the ovum. It has been proposed that PDC-109 also binds to carbohydrate moieties of glycoproteins present in the epithelia of oviduct and based on this it has been suggested PDC-109 helps in the formation of sperm reservoir in the oviduct (Ignotz *et al.*, 2001).

Another recent work shows that under *in vitro* condition PDC-109 exhibits chaperone-like activity against a variety of target proteins, which could be modulated by phospholipid binding. These observations suggested that PDC-109 may function as a molecular chaperone *in vivo* and help in maintaining other seminal plasma proteins in a functionally active, folded form (Figure 8) (Sankhala and Swamy, 2010).

6. Fibronectin type II Domain

Fibronectin is a large multi-domain glycoprotein, which is present in soluble form in plasma and in insoluble form in the extracellular matrix. Fibronectin binds to collagen, fibrin, heparin, DNA and actin. It provides the network of anchorage for binding of cell surface receptor, so it plays important role in cell migration and maintenance of tissue integrity. (Dean *et al.*, 1987; Mosher, 1993). Major part of the sequence of fibronectin consists of three types of repeats, which are called type I, type II and type III. Type II modules (FnII modules) are small, compact two-disulphide-bond domains of about 40 amino acid residues and it is duplicated. This domain is part of collagen binding region of fibronectin. Type II domains are also present in various other protein with diverse function, including seminal plasma protein PDC-109, BSP A-3, members of the mannose receptor–phospholipase A2 receptor family, mannose-6-phosphate receptors, pancreas-specific sel-1 proteins of vertebrates, matrix metalloproteinases MMP-2 and MMP-9, blood coagulation factor XII, and hepatocyte growth factor activator (Constantine *et al.*, 1992; Skorstengaard *et al.*, 1994).

NMR and crystal structures are known for the PDC-109, second FnII module of PDC-109, the two FnII domains of fibronectin, and the three FnII domains of gelatinase A/MMP-2. From structural analysis it is known that the FnII domains consists of 40-60 amino acids with two antiparallel beta sheets, a conserved core tryptophan residue and two disulfide bonds between cysteine 1-3 and 2-4 (Constantine *et al.*, 1992; Sticht *et al.*, 1998; Wah *et al.*, 2002). Solution structure of domain b of PDC-109 and its superimposed structure with FnII module of fibronectin are shown in Figure 9 and 10, respectively.

Although FnII domains are widespread in occurrence in diverse protein of vertebrates, their presence has not been reported in invertebrates, including the completely sequenced genome of *Caenorhabditis elegans* and *Drosophila melanogaster*. One interesting example is a transmembrane protein SEL-1, which is proposed to have a role in protein turnover, contains FnII domain. However, its orthologs sel-1 in invertebrates do not have this domain (Harada *et al*., 1999). It has been suggested that FnII domain has evolved from the more ancestral kringle domain by major structural modification (Ozhogina *et al*., 2001).

Based upon sequence similarity in FnII domain and kringle domain of proteases it has been proposed that they are divergent members of same fold family (Patthy *et al.,* 1984). Analysis of these two domain shows that FnII domain has many similarities with the protein-fold of protease kringles. FnII domains are similar to kringles in as much as they are also characterized by two short antiparallel β-sheets and an exposed aromatic-rich ligand binding site, as well as two cystine bridges in close, quasi-orthogonal juxtaposition (Briknarova *et al.*, 1999, Ozhogina *et al.*, 2001).

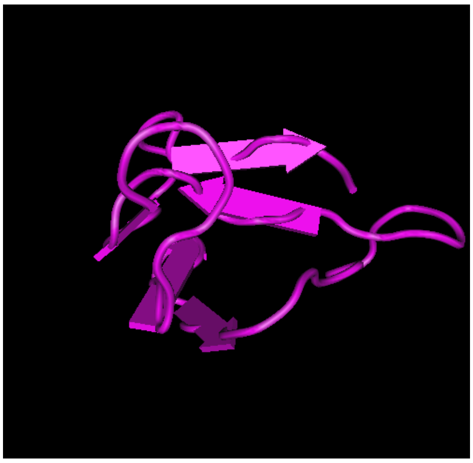


Figure 9. Ribbon drawing (stereo view) of PDC-109/b backbone fold. The antiparallel β sheets are indicated by arrows. Source: NCBI, MMDB ID: 57086.

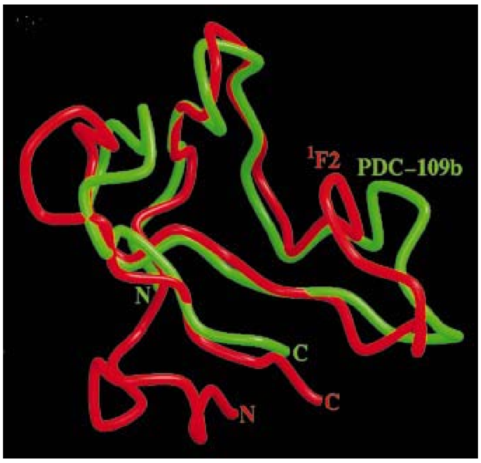


Figure 10. Comparison of the FnII modules of fibronectin (red) and PDC-109 domain b (green). The modules were superimposed over the backbone heavy atoms (N, Cα and C) of their common secondary structural elements. Reproduced from: *Structure* 5, 359–370.

7. Objective and major finding of present study

7.1 Importance of core tryptophan in FnII domain proteins

The major protein of bovine seminal plasma, PDC-109, has been identified several decades ago and it is among the most studied and well characterized seminal plasma proteins in mammals. The crystal structure of PDC-109 bound with ligand phosphorylcholine shows that a core tryptophan residue in each FnII domain binds to the choline head group with cation-π interaction. Also, in multiple sequence alignment of other known FnII domains of various proteins, it has been found that the core tryptophan is conserved in all of them. More importantly core tryptophan residues are also conserved in kringle domain, which are ancestral protein of FnII domains. These observations prompted us to investigate the importance of these residues in the activity of PDC-109.

Therefore cloning, expression and purification of PDC-109 was done in bacterial system. After characterization of rPDC-109, it was found that it has folded properly and its intrinsic fluorescence spectra in the absence and presence of various ligands were similar to wild type PDC-109. DSC thermograms also show that its thermal stability in the absence and presence of ligand is similar to that of the wild type protein, and CD spectral analysis shows that both secondary and tertiary structures of rPDC-109 are similar to those of the wild type protein. After confirming that the cloned protein is folded properly, various mutants were prepared. Mutational analysis showed that indeed the core Trp is important for PDC-109 function, as these single mutations resulted either in drastic decrease or complete loss of the activity of PDC-109.

There is high probability, these conserved trp is important in other FnII domain as well. Future studies in this direction can be very useful for many biomedical application, as FnII domain is present in other proteins, some of them plays important role in various fundamental biological process in normal as well as in disease altered condition.

7.2 Effect of spermine and spermidine on the lipid binding and chaperone like activity of PDC-109

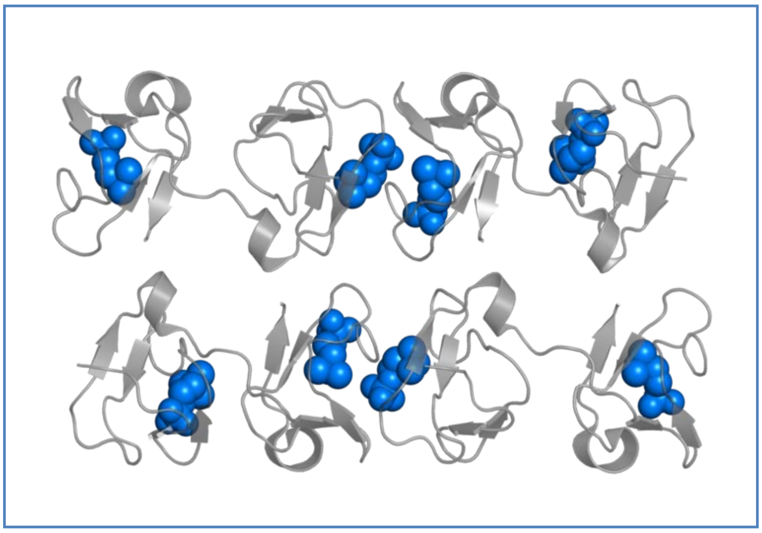
Spermine is a component of seminal plasma of many mammalian species. Various studies have been done to understand the role of spermine in reproduction. Previous studies have shown that, spermine binds to sperm plasma membrane and in presence of heparin it is released from the sperm plasma membrane. It has been shown in some species that spermine affects sperm capacitation. As spermine and PDC-109 both bind to sperm plasma membrane and play a role in sperm capacitation, studies have been carried out to investigate the effect of spermine and its precursor spermidine on lipid binding and chaperone like activity of PDC-109. Results from these studies show that in the presence of Spm/Spd lipid efflux induced by PDC-109 increases. Presence of Spm/Spd or PDC-109 prevents the aggregation of target proteins and help them to retain the activity under thermal stress. When Spm or Spd was used in combination with PDC-109, the observed increase in CLA was found to be more than the increase expected for a simple additive effect. In other words, PDC-109 and the polyamines appear to act in a synergistic manner in protecting target proteins from denaturation. The effect of Spm on both CLA and lipid efflux inducing ability of PDC-109 was found to be higher than Spd.

7.3 Effect of molecular crowding on PDC-109 function

Another important part of the present work is to study the effect of molecular crowding on the PDC-109 function. Under *in vivo* condition molecular crowding is one of the important factors, which affects most of the biological process. A growing number of studies suggest that crowding environment significantly affect the biological process in various ways. Therefore it is important to study the behaviour of a protein molecule mimicking *in vivo* environment. Many proteins are known to perform more than one function in biological context and it is known as protein moonlighting. However no previous study has been reported for effect of molecular crowding on two different function of same protein. To understand the effect of crowding on the lipid binding and CLA activities of PDC-109, in present study dextran (Mr-70,000) was used as a crowding agent. For investigation on lipid efflux ability of PDC-109, we used erythrocyte lysis assay and interaction study of PDC-109 with DMPC model membrane using SEM. Further, ITC study with DMPC model membrane and PDC-109 was performed. Results obtained from all these experiments show under crowding condition lipid efflux ability of PDC-109 increases significantly. To investigate the effect of crowding on CLA of PDC-109, the ability of PDC-109 to protect target protein G6PD and ADH from heat induced inactivation or aggregation was monitored in the absence and presence of dextran at 48 °C. The result show that in crowding condition, the CLA of PDC-109 reduced significantly. In conclusion, these findings clearly demonstrate crowding condition can affect different functions of same protein in different ways.

Chapter 2

Significance of Glycosylation and Conserved Core Tryptophan Residues in the FnII Domains of PDC-109 for Choline Phospholipid Binding and Chaperone-Like Activities



Four copies of PDC-109 bound with phosphorylcholine. The protein is shown in light gray colour and phosphorylcholine molecules are shown in blue colour. Source: EMBL-EBI. **PDB ID - 1H8P**

1. Abstract

The major protein of bovine seminal plasma, PDC-109 has two fibronectin type II (FnII) domains. The FnII domains bind to choline phospholipids with high specificity, which plays an important role in lipid efflux at the time of capacitation and are therefore, important for successful fertilization. Previous work has shown that PDC-109 acts as a molecular chaperone by protecting target proteins from various denaturing conditions. Multiple sequence alignment of FnII domains of different proteins shows that a core tryptophan is highly conserved in them. Crystal and solution structures of PDC-109 and its domain b, respectively suggested that this core tryptophan is important for ligand-binding. However, the significance of conserved tryptophan residues in FnII domains is not understood. In the present work two important aspects of PDC-109 have been studied: (1) the role of glycosylation, and (2) the importance of the conserved tryptophan residue, in chaperone-like activity (CLA) and lipid-binding ability of PDC-109. The present results show that glycosylation increases the chaperone-like activity and decreases lipid-binding ability of PDC-109. Mutation of tryptophan 47 in FnII domain 1 to alanine (W47A) resulted in a drastic decrease in its ligand-binding ability together with a ~50% decrease in the chaperone-like activity, whereas mutation of W93 and W106 in FnII domain 2 to alanine resulted in the complete loss of both ligand-binding and chaperone-like activities. These results demonstrate that the conserved tryptophan residues in PDC-109 are crucial for both choline phospholipid-binding and chaperone-like activities of this protein.

2. Introduction

Among all the mammalian seminal plasma proteins that have been investigated so far, the bovine seminal plasma proteins have been studied in the greatest detail. However, the role of glycosylation in PDC-109 has not been investigated previously. Glycosylation is one of the most common and widely observed forms of post translational modifications (Bechor and Levy, 2008). Studies on glycosylated proteins show their widespread presence in all domains of life (Spiro, 2002). In eukaryotes, with very few exceptions, all the membrane-associated and secretory proteins are glycosylated (Parekh, 1991). Protein glycosylation plays important roles in a variety of processes ranging from protein trafficking, folding, stability, turnover and quality control to the involvement in a large number of biological recognition events (Vik *et al.*, 2009; Moremen *et al.*, 2012). As the covalent addition of carbohydrate significantly influences protein function and properties, it is interesting to investigate the role of glycosylation in PDC-109 on its function.

Another major objective of the present study is to understand the importance of a core tryptophan residue that is highly conserved across all fibronectin type II (FnII) domains. As mentioned in Chapter 1, PDC-109 is composed of two tandem repeats of FnII domains and presence of this domain is fairly widespread among diverse vertebrate proteins. An earlier study showed that the FnII domain has evolved by a major structural change from a more ancient kringle domain, which is present both in vertebrates and invertebrates (Patthy *et al.*, 1984; Baker, 1985; Ozhogina *et al.*, 2001). Interestingly multiple sequence alignment shows that the core tryptophan, which is highly conserved in all other BSP proteins and FnII domains of many other proteins, is also conserved in its ancestral kringle domain, from which the FnII domain has evolved (Figure 1), suggesting that it is important for their function. Based on the crystal and solution structure of PDC-109 and other FnII domain proteins, it has been suggested that the core tryptophan is important for ligand-binding (Wah *et al.*, 2002; Constantine *et al.*, 1992). However, to date no systematic study on the role of the conserved core tryptophan residue in the activity of the FnII domain has been performed. Some of the proteins which contain FnII domains in their structure play crucial roles in many fundamental biological processes such as cell signalling, cell movement, modulation of cell adhesion, proliferation and differentiation etc (Baker, 1985; Banyai *et al.*, 1994; Pickford *et al.*, 1997; Sticht *et al.*, 1998; Napper *et al.*, 2006). In the present study, site-directed mutagenesis of the core tryptophan residues in the FnII domains was carried out in order to understand their importance in PDC-109. In view of the fact that the same residues are also conserved in other FnII domains, results obtained in this study are expected to provide insights into the structural basis of FnII domain function.

3. Materials and methods

3.1 Materials

Choline chloride and guanidine hydrochloride were purchased from Sigma (St. Louis, MO). Sephadex G-50 (superfine) and DEAE Sephadex A-25 were obtained from Pharmacia (Uppsala, Sweden). 1-Lyso phosphatidylcholine (Lyso PC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were purchased from Avanti Polar Lipids (Alabster, AL). Alcohol dehydrogenase (ADH), dithiothreitol (DTT), tris base, ethylenediaminetetraacetic acid (EDTA), isopropylthiogalactoside (IPTG), glutathione (GSH), and other chemicals were purchased from local suppliers and were of the highest purity available. PDC-109 was purified as described earlier by gel filtration on sephadex G-50 followed by affinity chromatography on DEAE Sephadex A-25 (Calvete *et al.*, 1996; Ramakrishnan *et al.*, 2001). The purified protein was dialyzed extensively against 50 mM tris buffer, 0.15 M NaCl, 5 mM EDTA, 0.025% sodium azide, pH 7.4 (TBS-I) and stored at 4°C.

3.2 Cloning, expression and purification of wild type and mutant PDC-109

Total RNA was isolated from bovine seminal vesicle tissue using TRIzol reagent and cDNAs were made from total RNA, using SuperscriptTM first strand synthesis kit. PDC-109 was PCR-amplified from the cDNAs using the forward primer 5’-*ACTCATATGGATCAGGACGAAGGTGTTTC*-3’ and reverse primer 5’-*GTTTAAGCTTCTAGCAATACTTCCAAGCTCTG*-3. The PCR amplicon was cloned into pET21a vector (Novagen, Madison, WI, USA) using the restriction sites present in the primers and the vector. The sequence of the PDC-109 pET21a construct was verified and found to be identical to that reported earlier. Since we used a bacterial system for the cloning, an additional methionine (M) residue was incorporated in PDC-109 sequence prior to N-terminal aspartic acid. PDC-109 was expressed in *E. coli* BL-21 cell line.

PCR-based site-directed mutagenesis was performed to make specific mutants. For making a single mutant, PDC-109 expression plasmid pET21a was used as the template. The tryptophan residues at positions 47, 93 and 106 were replaced individually by alanine, using the following forward (FP) and reverse (RP) primers: FP 5 -*TCCTTATTCCCGGCGTGTTCCCTCG*-3’ and RP 5’*CGAGGGAACACCACGGGAATAAGGA*-3’ for W47A, FP 5’-*TGTGGATGTCTGCGTGCTCACTCTC*-3’ and RP 5’-*GAG AGT GAG CAC GCA GAC ATC CAC A*- 3’ for W93A, and FP 5`-*AAGGACAGAGCTGCGAAGTATTGCTAG-* 3’ and RP 5’-*CTAGCAATACTTCGCAGCTCTGTCCTT-* 3’ for W106A substitution. All mutations were confirmed by DNA sequencing. The mutated proteins were expressed in *E. coli* BL21. Expression profiling of cloned proteins suggested the presence of protein in inclusion bodies. Hence large scale cultures were set up and inclusion bodies were collected for further processing as described below.

3.3 Refolding of PDC-109 using GSH/GSSG redox system

Inclusion bodies containing misfolded PDC-109 as the major protein content were collected and washed thrice with TBS-I containing 1 mM DTT, 0.05% Triton X-100 and 1 M urea. After vigorous washing, final inclusion bodies were collected and dissolved in TBS-I containing 10 mM DTT and 6 M guanidine hydrochloride (GdnHCl). Refolding was initiated by dropwise addition of the above solution into the refolding buffer (TBS-I) containing 1.4 M GdnHCl, 2 mM GSH and 0.4 mM GSSG redox system. Refolding reaction was carried out at 4 °C for 18 hrs. After completion of the reaction, solution containing the recombinant protein with wild type sequence was dialyzed against TBS-II buffer containing 25 mM tris, 1 M NaCl, 0.025% sodium azide at pH 6.4, while mutant proteins were dialyzed against the same buffer, but with a salt concentration of 0.1 M. After dialysis, the solution was centrifuged at 12000 rpm for 30 minutes. The supernatant was collected and loaded on DEAE-Sephadex A25 for affinity purification. Further purification procedure was similar to that described for wild type PDC-109 (Ramakrishnan *et al.*, 2001; Sankhala *et al.*, 2011). Protein concentrations were estimated by the Bradford assay using BSA as the standard (Bradford, 1976). Purity of recombinant PDC-109 (rPDC-109) as well as the mutant proteins was verified by SDS-PAGE from coomassie blue-stained or silver-stained gels.

3.4 Differential scanning calorimetry

Differential scanning calorimetric (DSC) measurements were performed on a MicroCal VP DSC apparatus (MicroCal LLC, Northampton, MA, USA). rPDC-109 solution in TBS-I, at a concentration of 0.6 mg/mL, was heated from 10 to 90°C at a scan rate of 30°/hour under a constant pressure of 23.4 psi. The contribution of the protein to the calorimetrically measured heat capacity was determined by subtracting buffer-buffer baseline from the sample data prior to analysis. Experiments aimed at investigating the effect of phosphorylcholine (PrC) binding on the thermal stability and oligomeric status of rPDC-109 were carried out under similar conditions and the data obtained were analyzed using the '*non two-state mode of fitting*' in MicroCal Origin software.

3.5 Circular dichroism spectroscopy

CD spectral studies were performed using a JASCO J-815 spectropolarimeter. Spectra were recorded using a 0.2 cm path length quartz cell. Protein concentrations of 0.1 and 0.6 mg/ml were used to record the far- and near-UV CD spectra of WT PDC-109, rPDC-109 as well as its mutants. To probe the effect of PrC-binding on the protein conformation, wild-type PDC-109, rPDC-109 and the mutants were preincubated with 10 mM PrC and ellipticities were recorded as a function of wavelength at 25 ^{o}C. Each spectrum reported was the average of 10 consecutive scans from which buffer scans, recorded under the same conditions, were subtracted. The observed ellipticity values were converted to mean residue ellipticities (MRE).

3.6 Preparation of liposomes

Lipids dissolved in dichloromethane in a glass test tube were dried under a gentle stream of nitrogen gas and the final traces of the solvent were removed by vacuum desiccation for ca. 3 h. The dried lipid film was hydrated by vortexing for 5 min in TBS-I to give the desired lipid concentration. Small unilamellar vesicles (SUVs) were prepared by sonication of the lipid suspension in a bath sonicator for 30 min at room temperature.

3.7 Fluorescence spectroscopy

Steady state fluorescence measurements with rPDC-109 and mutants were performed using an ISS PC1 photon-counting fluorescence spectrometer (Champaign, IL, USA) at room temperature, with the excitation and emission bandpass filters set at 2 and 5 nm. All fluorescence studies were carried out on protein samples in TBS-I buffer with $OD_{280nm} \leq 0.1$ (≤ 3.0 μM). Protein samples were excited at 280 nm and emission spectra were recorded between 300 and 400 nm. Measurements in the presence of different lipids were performed at a lipid concentration of 0.15 mM (Which corresponds to a lipid/PDC-109 ratio ≈50), whereas measurements in the presence of PrC were made at a resultant ligand concentration of 20 mM (corresponding to a ligand/protein ratio ≈6600). Previous studies have shown that at these concentrations, the choline-binding sites of PDC-109 are mostly occupied by PrC or choline phospholipid (DMPC, Lyso-PC) (Anbazhagan and Swamy, 2005; Anbazhagan *et al.*, 2011).

In bis-ANS-binding studies, a 10 μM solution of bis-ANS was prepared from a 890 μM stock solution and added to 140 μg (10 μM) of proteins in a final volume of 1 ml. After a one-minute incubation to ensure proper binding, the samples were excited at 390 nm and emission spectra were recorded from 400 nm to 600 nm with the excitation and emission band passes set at 2 and 3 nm, respectively.

3.8 Disruption of erythrocyte cell membrane by wild type and mutant PDC-109

The ability of wild type and mutant PDC-109 to disrupt erythrocyte cell membrane was investigated by confocal microscopy or by monitoring the release of haemoglobin from erythrocytes, due to lysis resulting from PDC-109 binding.

3.8a Confocal microscopy - The effect of PDC-109 binding to the plasma membrane on the shape and integrity of erythrocytes was investigated by confocal microscopy. Imaging was done in the transmission mode using a Leica TCS SP2 confocal microscope (Heidelberg, Germany). Human erythrocytes (0.1 %) in TBS-I buffer were used for control experiments, whereas to monitor the effect of interaction of WT PDC-109, rPDC-109 and mutants with erythrocyte membranes, a 0.1 % suspension of erythrocytes was prepared with 25 µg/ml of the appropriate protein. At regular time intervals, about 50 µl of the erythrocyte suspension, which was incubated with each of the proteins was directly spotted on a clean glass slide, covered with a cover slip and then transferred to the confocal stage for imaging.

3.8b Measurement of haemoglobin release from erythrocytes after PDC-109 binding - The ability of PDC-109 to disrupt the plasma membrane of human erythrocytes was investigated by monitoring the release of haemoglobin, resulting from lysis of erythrocytes upon incubation with PDC-109. To check the effect of glycosylation, different amounts of WT and rPDC-109 (50, 75, 100 and 125 µg) were added into TBS-I buffer and the volume was then adjusted to 800 µl. For mutant proteins, the reaction was carried out at a fixed concentration of 100 µg/ml. Reaction was started with the addition of 200 µl of 4% RBC. The mixture was then incubated for 1 hour at room temperature. After incubation, the samples were centrifuged at 3000 rpm for 10 minutes, the supernatant was collected and its optical density was monitored at 415 nm (which corresponds to absorption by the haem moiety of haemoglobin) using a Perkin-Elmer double-beam spectrophotometer.

3.9 Chaperone-like activity

The chaperone-like activity (CLA) of rPDC-109 and the mutants was investigated by monitoring their ability to prevent heat-induced aggregation of yeast alcohol dehydrogenase (ADH) either by a spectrophotometric (aggregation) assay or by atomic force microscopy (Sankhala and Swamy, 2010; Asthana *et al.*, 2012) .

3.9a Aggregation assay - This assay was done as follows. Samples containing different ratios of chaperone (WT and rPDC-109) to yeast ADH (100 µg/ml) were incubated at room temperature for about 2-3 minutes and the final volume was then made up to 1 mL using 50 mM Tris buffer (pH 7.4), containing 100 mM NaCl. Aggregation was monitored at 48 °C by recording light scattering at 360 nm as a function of time using a Perkin Elmer UV-Vis spectrophotometer. For mutant proteins, CLA was checked with a fixed target to chaperone ratio (1:1.5 w/w).

3.9b Atomic force microscopy – ADH samples (40 µg/mL) in TBS-I buffer were incubated in the presence or absence of 20 µg/mL PDC-109 at 48 °C in a water bath for 50 min and then transferred to room temperature. Samples for AFM studies were prepared as described above. An AFM image of native enzyme (stored at 4°C) was used as a control. A 50-80 µL aliquot of the protein solution was carefully deposited on a freshly cleaved mica sheet (1 cm × 1 cm) and allowed to dry for 20-30 min, rinsed with HPLC grade water, dried, and transferred to the AFM stage. Imaging was performed in semi-contact mode using a SOLVER PRO-M atomic force microscope (NT-MDT, Moscow, Russia), equipped with a 10.0 µm bottom scanner. NSG10 cantilevers with Au reflective coating and a nominal spring constant of 11.8 N/m were used for the scanning. Force was kept at the lowest possible value by continuously adjusting the set point and feedback gain during imaging. Images were analyzed using NOVA software, supplied by NTMDT.

3.10 Glutaraldehyde cross-linking

Glutaraldehyde cross-linking reaction was carried out by adding 5 µL of 1% glutaraldehyde to 50 µg of rPDC-109 or mutant proteins in phosphate buffer (pH 7.5) in a final volume of 100 µl. The reaction was allowed to proceed for five minutes and was terminated by the addition of 10 µL of 1 M Tris-HCl buffer, pH 8.0. Cross-linked protein samples were solubilized in equal volume of Laemmli buffer and electrophoresis was performed on 15% SDS-polyacrylamide gels (Laemmli, 1970).

4. Results

4.1 Multiple sequence alignment shows core tryptophan is conserved in FnII domains

Multiple sequence alignment of all known FnII domains from different proteins, which exhibit versatile functions, shows that a core tryptophan residue is highly conserved in them. Moreover, these tryptophan residues are also conserved in the evolutionarily more ancient proteins from which the FnII domains have evolved (**Fig. 1**).

```
PDC-109A  ------EECV-----FPFVY----------RNRKHFDCTVHGSL---FPWCSLDADYV--GRWKY-CAQR
PDC109B   ---RDYAKCV-----FPFIY----------GGKKYETCTKIGSM-WM-SWCSLSPNYDKDRAWKY-C---
BSPA3a    ------NKCV-----FPFIY----------GNKKYFDCTLHGSL---FLWCSLDADYT--GRWKY-CT--
BSPA3b    ----DYAKCV-----FPFIY----------EGKSYDTCIKIGST-FMNYWCSLSSNYDEDGVWKY-C---
BSPH1a    ----TDGECV-----FPFHY----------KNGTYYDCIKSKAR---HKWCSLNKTYE--GYWKF-CSA-
BSPH1b    ---EDFANCV-----FPFWY----------RRLIYWECTDDGEA-FGKKWCSLTKNFNKDRIWKY-CE--
HSPa      STRKPDNKCV-----FPFKY----------QGRQYYDCTRTDSF---HRWCSLTENYS--GKWRY-CV--
HSPb      --AEDYAKCF-----FPFVY----------RG-TYHTCTTDGSF-FLIPWCSVTPNYDRDGGWKY-CM--
BSPPa     -------ECV-----FPFHY----------KNGTYYDCIKSKAR---HKWCSLNKTYE--GYWKF-CSAE
BSPPb     -----------------FWY----------RRLIYWECTDHGEA-FGRKWCSLTKNFNKDRIKWY-CE--
BSPM      ---------------FPFWY----------RHMIYWDCTEDGEV-FGKKWCSLTPNYNKDQVWKY-CIE-
1Fn2      GGNSNGEPCV-----LPFTY----------NGRTPYSCTTEREQ-DGHLWCSTTSNYEQDQKYSF-CTDH
2Fn2      GGNSNGALCH-----FPFLY----------NNHNYTDCSTSEGRRDNMKWCGTTQNYDADQKFGF-CPMA
MMP2a     YGNADGEYCK-----FPFLF----------NGKEYNSCTDTGRS-DGFLWCSTTYNFEKDGKYGF-CPHE
MMP2b     GGNAEGQPCK-----FPFRF----------QGTSYDSCTTEGRT-DGYRWCGTTEDYDRDKKYGF-CPET
MMP2c     GGNSEGAPCV-----FPFTF----------LGNKYESCTSAGRS-DGKMWCATTANYDDDRKWGF-CPDQ
MMP9a     FGNADGAACH-----FPFIF----------EGRSYSACTTDGRSSDGLPWCSTANYDTDDRFGF-CPSER
MMP9b     DGNADGKPCQ-----FPFIF----------QGQSYSACTTDGRS-DGYRWCATTANYDRDKLFGF-CPTR
MMP9c     GGNSAGELCV-----FPFTF----------LGKEYSTCTSEGRG-DGRLWCATTSNFDSDKKWGF-CPDQ
ManR      LGNANGATCA-----FPFKF----------ENKWYADCTSAGRS-DGWLWCGTTTDYDTDKLFGY-CPLK
M6PR      PETDDGVPCV-----FPFIF----------NGKSYEECIIESRA---KLWCSTTADYDRDHEWGF-CRHS
HGFA      ALTEDGRPCR-----FPFRY----------GGRMLHACTSEGSA-HR-KWCATTHNYDRDRAWGCYCVEA
PLA2R     KGNAHGTPCM-----FPFQY----------NQQWHHECTREGREDHNLLWCATTSRYERDEKWGF-CPDP
DEC-205   DGNSYGRPCE-----FPFLI----------DGTWHHDCILD--EDHSGPWCATTLNYEYDRKWGI-CLKP
ENDO180   QGNSHGKPCT-----IPFKY----------DNQWFHGCTSTGREDG-HLWCATTQDYGKDERWGF-CPIK
FXII      VLTVTGEPCH-----FPFQY----------HRQLYHKCTHKGRP-GPQPWCATTPNFDQDQRWGY-CLEP
NTK       NATNLGVPCLHWDEVPPFLERSPPASWAELRGQPHNFCRSPDGA-G-RPWCFYRNAQ-GKVDWGY-CD--
CRorK     AQTSSGKQCAPWIDSTSRDF--NVHRFPE-LMNSKNYCRNPGGK-KSRPWCYSK-PM---GQEEY-CDVP
Pgnk4     STTTTGKKCQSWSSMTPHRHQKTPENYPN-AGLTMNYCRNPDAD-K-GPWCFTTDPS---VRWEY-CNLK
```

Figure 1. Multiple sequence alignment of FnII modules of various proteins. Shown from top to bottom are bovine seminal plasma protein PDC-109 (PDC-109a and PDC-109b); Bovine seminal plasma protein BSPA3 (BSP A3a and BSP A3b); Binder of sperm protein human (BSPH1a and BSPH1b); Binder of sperm protein from horse (HSPa and HSPb); Binder of sperm protein from pongo (BSPPa and BSPPb); Binder of sperm protein from mus musculus (BSPM); fibronectin (1Fn2 and 2Fn2); matrix metalloproteinases 2 (MMP2a, MMP2b and MMP2c) and 9 (MMP9a, MMP9b and MMP9c); macrophage mannose receptor (ManR); cation-independent mannose-6-phosphate receptor (M6PR); hepatocyte growth factor activator (HGFA); phospolipase A2 receptor (PLA2R); phagocytosis receptor of human dendritic cell (DEC-205); endocytic transmembrane glycoprotein Endo180 (Endo180); blood coagulating factor XII (FXII); the kringle domain of the rat neurotrypsin (NTK); the kringle domain of *C. elegans* Ror-type receptor tyrosine kinase (CRorK); the fourth kringle domain of human plasminogen (PgnK4). The alignment was generated using Clustal Omega programme.

.

4.2 Cloning, expression, refolding and purification of PDC-109

PDC-109 was cloned in pET21a vector and expressed in *E. coli* BL-21 DE3 cell line. The expression profile suggests that PDC-109 does not exist in soluble fractions but precipitates out as inclusion bodies (**Fig. 2**), probably because of the presence of multiple disulphide bonds in the protein structure. The misfolded protein from the inclusion body was refolded using the GSH/GSSG redox system. Preliminary experiments where the concentration of various components were varied suggested that refolding buffer containing 1.4 M GdnHCl, 2 mM GSH and 0.4 mM GSSG gave the best refolding. Refolded protein from the above solution was purified using the standard PDC-109 purification procedure, described in section 3.2.2 (Calvete *et al.*, 1996; Ramakrishnan *et al.*, 2001).

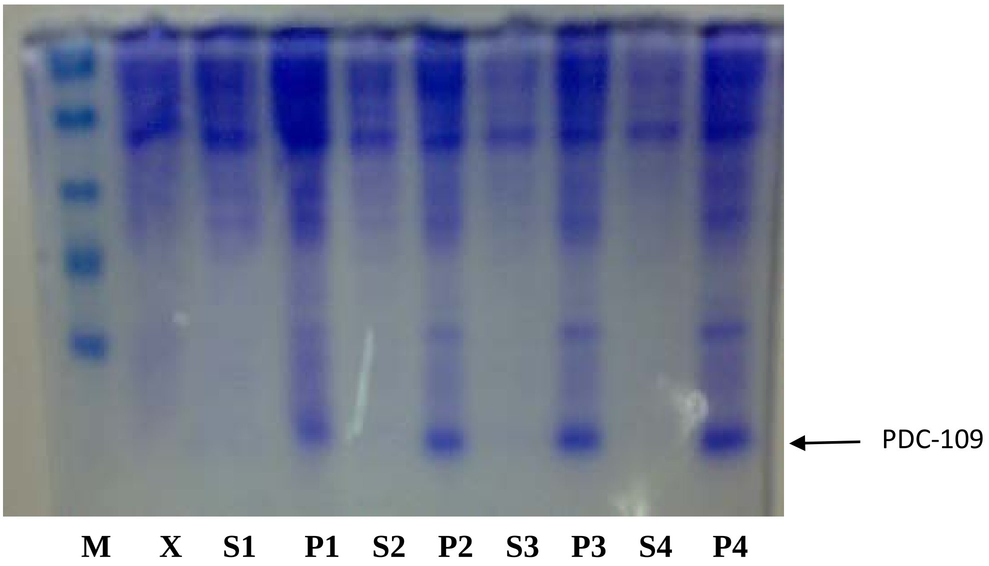


Figure 2: Protein expression profiling. The lanes indicate different samples as described. **M**, markers; **X**, cell lysate before IPTG induction; **S1**, **S2**, **S3** and **S4** are supernatants of cell lysates after IPTG induction, after 1, 2, 3 and 4 hours, respectively. **P1, P2, P3 and P4** are corresponding pellets.

4.3 WT and rPDC show similar fluorescence spectra with ligand binding

Fluorescence spectra of WT and rPDC-109 were recorded in the native state and in presence of various ligands. In the native condition fluorescence maximum was observed at 349 nm for both WT and rPDC-109. In the presence of PrC, Lyso PC and DMPC, fluorescence maximum is blue shifted with increase in fluorescence intensity. The shift in emission maximum and increase in fluorescence intensity are comparable for WT and rPDC-109 (**Fig. 3**).

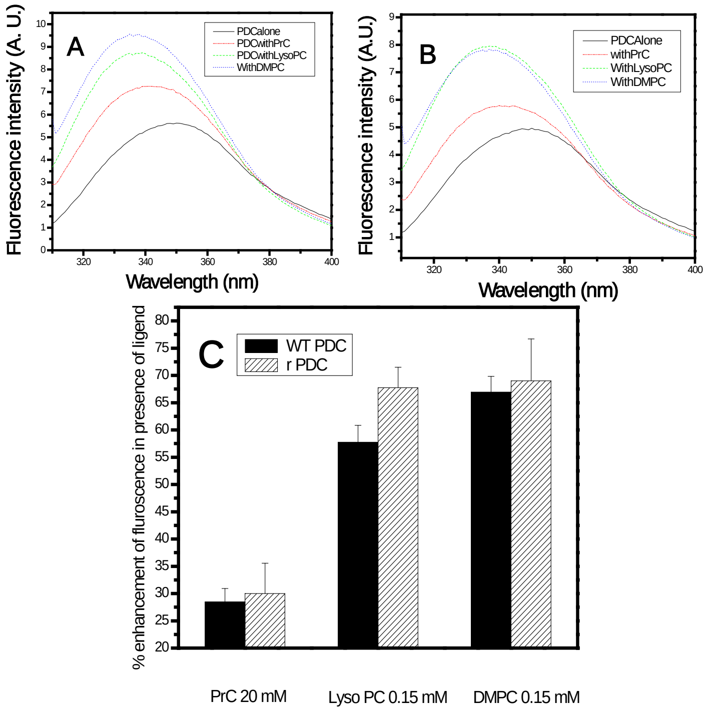


Figure 3. Fluorescence spectra of (A) WT and **(B)** rPDC-109 obtained in the native state and in the presence of 20mM PrC, 0.15mM Lyso-PC and 0.15mM DMPC. **(C)** Bar diagram representation of percent enhancement in fluorescence intensity after binding with various ligands.

4.4 Thermal denaturation of rPDC-109 in the absence and presence of PrC

Thermal stability of rPDC-109 was investigated by DSC. Representative thermograms of rPDC-109 in the absence and presence of PrC are shown in Figs. 4A and 4B, respectively. The thermogram of rPDC-109 alone could be best fitted to a non-two-state model assuming the occurrence of two thermotropic transitions centred at about ~311 K and 326 K (**Fig. 4A**). Values of the ratio of calorimetric enthalpy to van't Hoff enthalpy ($\Delta H_c/\Delta H_v$) corresponding to these transitions are given in Fig. 3A. $\Delta H_c/\Delta H_v$ ratio at 311 K is fractional (0.25) which demonstrates the occurrence of oligomeric species, whereas this ratio is close to 1 at 326 K, indicating that this transition corresponds to the unfolding of the monomer. The calorimetric scans obtained in the presence of PrC could be assigned to a single entity which unfolds at ~343 K (**Fig. 4B**).

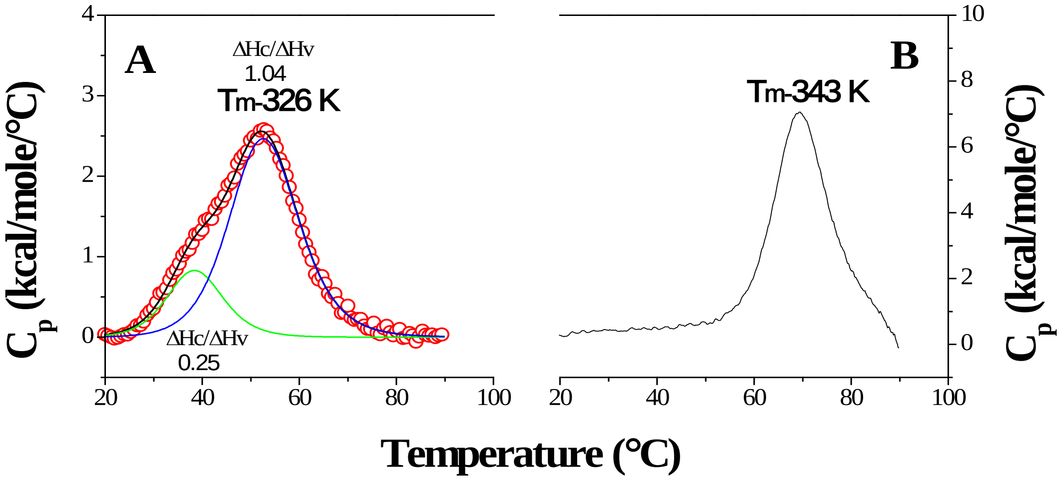


Figure 4. DSC thermograms of rPDC-109. (A) Protein alone and (B) in the presence of 20 mM PrC. Thermogram of rPDC-109 alone could be best fitted to a non-two-state model assuming the occurrence of two thermotropic transitions centred at about ~311 and 326 K

whereas the calorimetric scans obtained in the presence of PrC could be analyzed in terms of a single entity which unfolds at ~343 K.

4.5 Absence of glycosylation and substitution (W47A, W93A and W106A) minimally altered the structure of PDC-109

The effect of (lack of) glycosylation and different point mutations on the structure of PDC-109 was investigated by CD spectroscopy. It was observed that far- and near-UV CD spectra of recombinant (**Fig. 5**) and mutant PDC-109 (**Fig. 6**) do not differ significantly from that of the wild type protein. Further, analysis of the secondary structure content by the CONTIN analysis programme available in the Dichroweb website showed that the content of various secondary structural elements of rPDC-109 and its mutants is similar to that of the wild type protein (**Table 1**).

In the presence of PrC, the spectra of rPDC-109, W47A and W106A show moderate increase in ellipticity, which is similar to that obtained for the wild type protein. For W93A, in the presence of PrC, the ellipticity in both near- and far-UV CD spectra decreased slightly as compared to that of W93A protein alone.

Table 1: Secondary structure analysis of wild type, recombinant and mutant forms of PDC-109. Analysis was done by using CONTIN analysis programme available in the DICHROWEB website.

Protein	α-Helix	β-Sheet	Turns	Unordered
rPDC-109	6.2%	38.7%	21.9%	33.1%
W47A	6.6%	38.3%	22.1%	32.9%
W93A	6.3%	39.1%	22.1%	32.4%
W106A	6.7%	38.3%	22.4%	33.2%

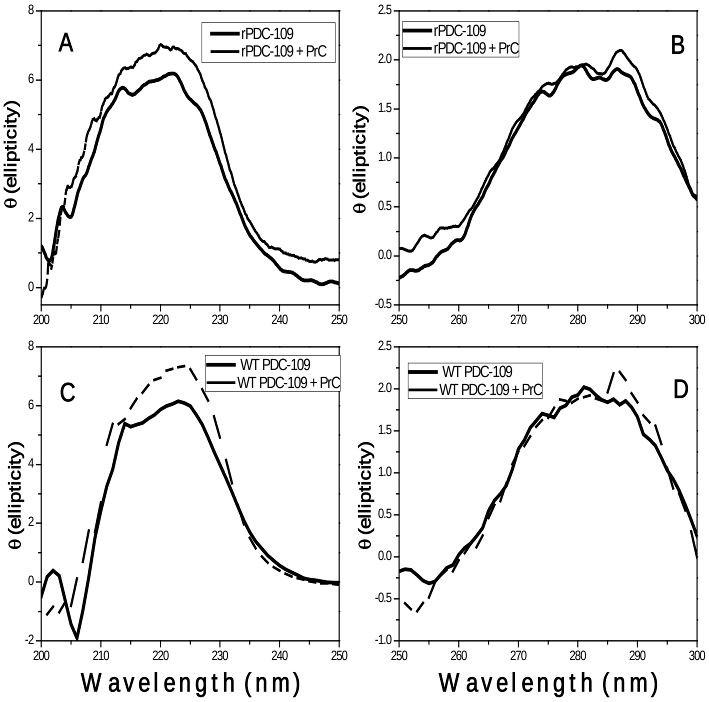


Figure 5. CD spectra of wild-type and recombinant PDC-109. (A,C) Far UV region, (B, D) near UV region. Spectra in A and B correspond to rPDC-109 and spectra in B and

D correspond to WT PDC-109. Solid lines, protein alone; dotted lines, protein pre-incubated with 10 mM PrC.

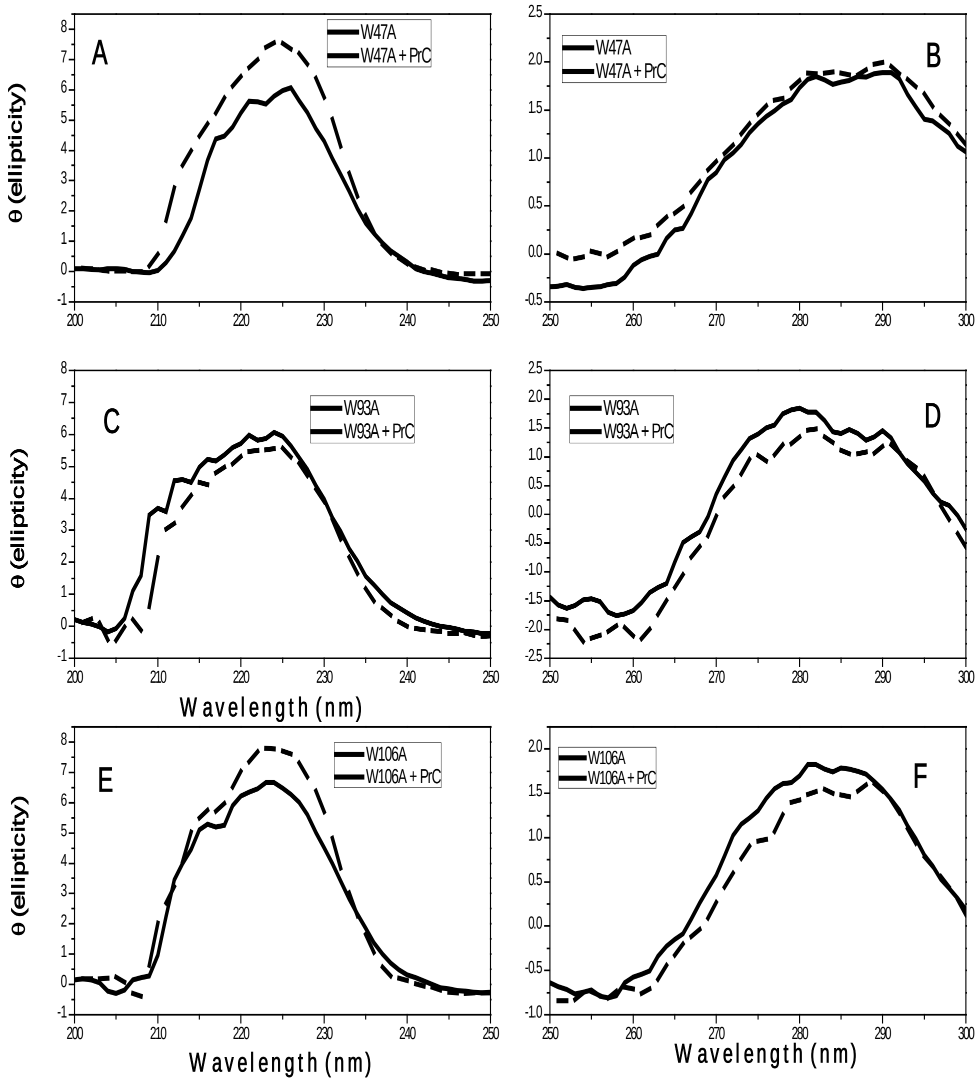


Figure 6. CD Spectra of mutant proteins. Solid lines show the spectra of protein alone, dashed line show spectra of protein preincubated with 10 mM PrC. Figures A, C and E show far-UV CD spectra of mutants W47A, W93A and W106A, respectively. Figure B, D and F show near-UV CD spectra of W47A, W93A and W106A, respectively.

4.6 W47A, W93A and W106A mutants show significant change in intrinsic fluorescence spectra of PDC-109 upon PrC binding compared to WT and rPDC-109

Fluorescence spectra of recombinant and mutant PDC-109 proteins in the absence and in the presence of 20 mM PrC are shown in (**Fig. 7)**. The fluorescence emission maximum of rPDC-109 is seen at 349 nm, which is blue shifted to 340 nm with about 24% increase in the fluorescence intensity in the presence of PrC, which is in good agreement with the results obtained for the binding PrC to WT PDC-109 (Anbazhagan *et al.*, 2008). Fluorescence emission maximum of W47A mutant in the absence of PrC was seen at 347 nm, which blue shifted to 340 nm in the presence of PrC with a 6% increase in the fluorescence intensity. For W93A and W106A mutants, emission maxima were seen at 349 nm and in the presence of PrC they shifted to 345 and 343 nm with very small changes (decrease) in intensity.

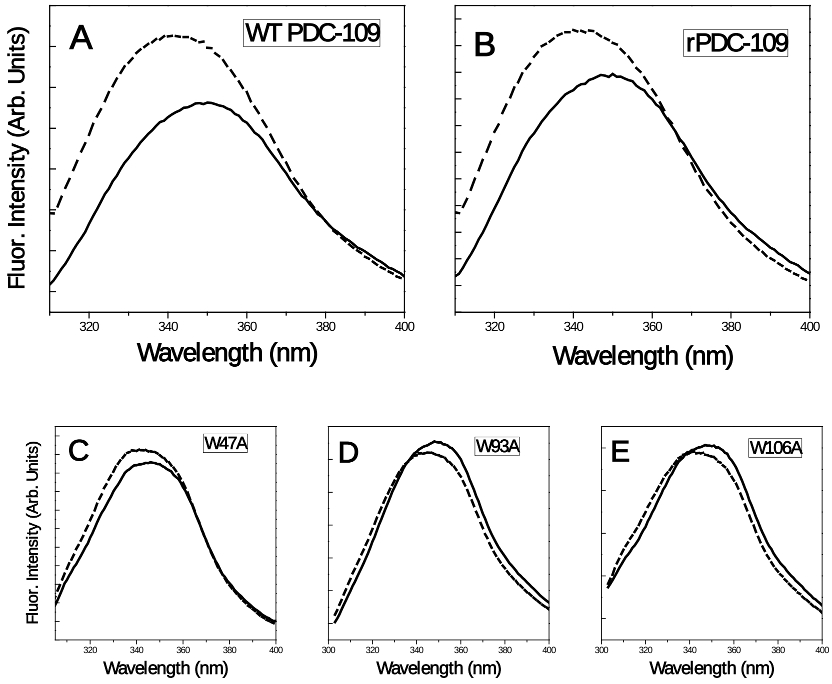


Figure 7. Fluorescence spectra of mutant PDC-109 in presence and absence of PrC. **(A)** WT PDC-109, **(B)** rPDC-109, **(C)** W47A, **(D)** W93A and **(E)** W106A. Solid lines show spectra in the absence of PrC and dotted lines show spectra in the presence of 20 mM PrC. All spectra were recorded with excitation wavelength at 280 nm and protein concentration used was 40 µg/ml.

4.7 Glycosylation of PDC-109 suppresses the lipid efflux ability of PDC-109 and tryptophan residues at positions 47, 93 and 106 are crucial for this ability

In previous work we characterized the binding of PDC-109 to erythrocytes using confocal microscopy. These studies showed that PDC-109-binding completely disrupted the erythrocyte membranes, leading to cell lysis and release of haemoglobin, which could be measured by absorption spectroscopy (Damai *et al.*, 2010). Therefore, in the present study we adopted the same approach to compare the relative abilities of rPDC-109 and the mutants W47A, W93A and W106A to bind to erythrocytes and disrupt their structure. In the absence of PDC-109, the erythrocytes had a well defined morphology with intact plasma membrane **(Fig. 8A).** In the presence of wild type PDC-109 the plasma membrane appears to have lost its integrity, as it was broken into smaller size fragments **(Fig. 8B)**, which is consistent with previous results (Damai *et al.*, 2010). Interestingly, under similar conditions, binding of rPDC-109 led to a near-complete disruption of the erythrocyte membranes **(Fig. 8C).**

The above observations were further confirmed by monitoring the lysis of human erythrocytes resulting from the binding of PDC-109. In this assay it was observed that the content of haemoglobin in the supernatant increased with increase in the concentration of PDC-109. At all concentrations of the protein investigated, the concentration of haemoglobin released was found to be significantly higher for rPDC-109 than for the wild type protein **(Fig. 8D),** suggesting that recombinant

PDC-109 is more efficient in inducing lipid efflux from the membrane than the wild type protein.

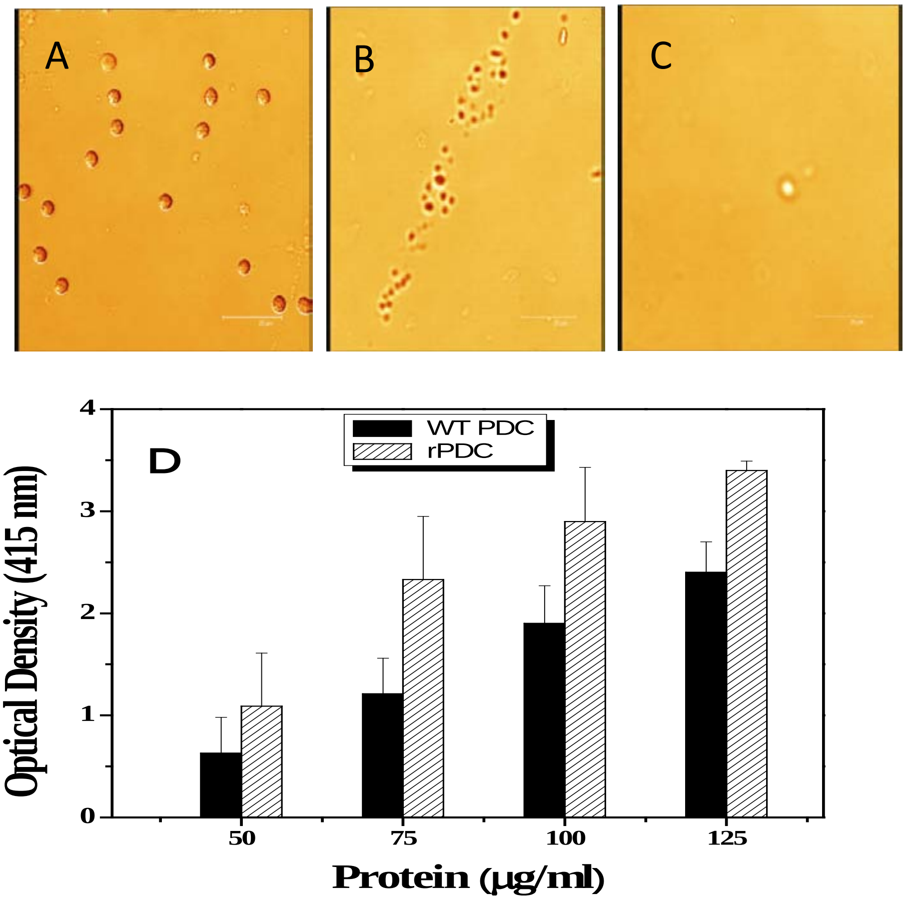


Figure 8. Confocal image of erythrocyte morphology after binding with PDC-109. (A) Control experiment, 0.1% human RBCs **(B)** 0.1% human RBCs with 25 µg wild type PDC-109 and allowed to interact for 15 minutes **(C)** 0.1% human RBCs with 25 µg cloned PDC-109 and allowed to interact for 15 minutes **(D) Erythrocyte lysis assay.** Absorption due to haemoglobin released upon lysis of 0.8 % erythrocyte solution at different concentrations of wild type and recombinant PDC-109 was monitored at 415 nm. Average values obtained from three independent experiments are shown. At all concentrations, the recombinant protein showed higher ability to disrupt erythrocyte plasma membrane than the wild type protein.

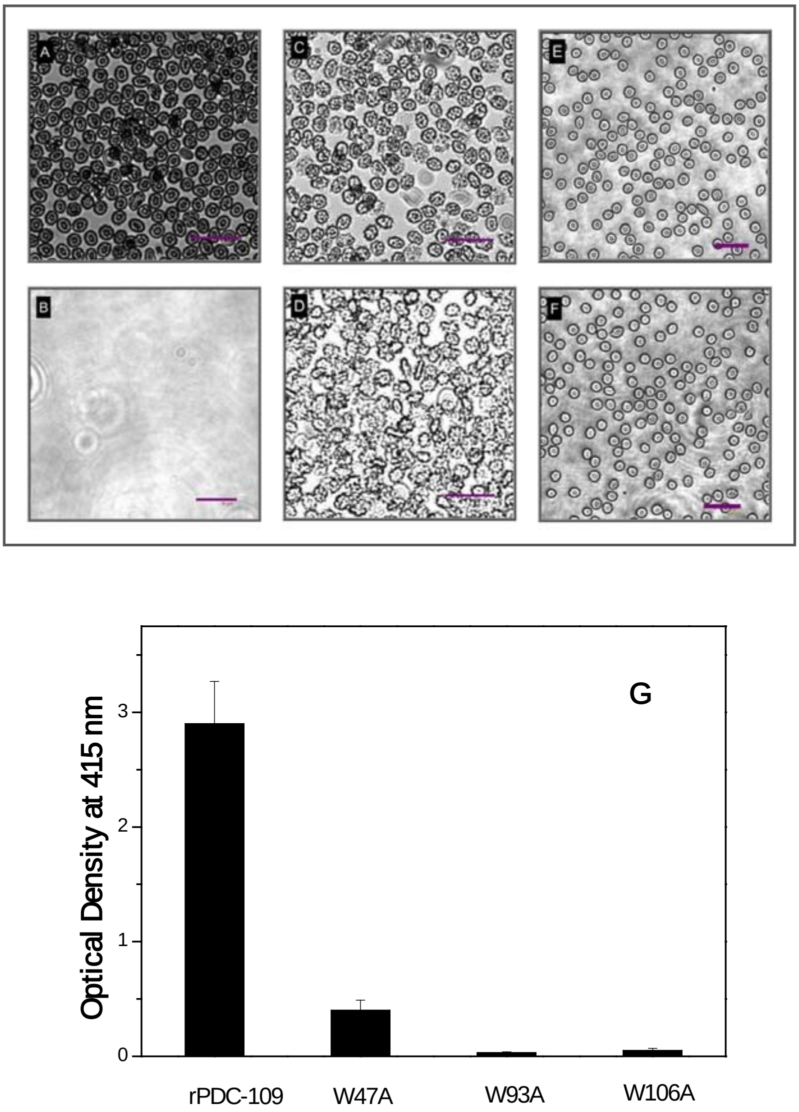


Figure 9. Confocal images of erythrocyte morphology after binding with PDC-109. Human erythrocytes (0.1 % in TBS) were used in all experiments. Protein concentration was 25 µg/ml for all experiments. **(A)** Erythrocytes in TBS, **(B)** upon incubation with rPDC-109 for 15 minutes, **(C,D)** upon incubation with W47A mutant for 30 and 60 minutes, respectively. **(E, F)** upon incubation for 60 minutes with W93A and W106A, respectively. Scale bar corresponds to 20 µm. **(G)** Disruption of erythrocyte membrane by rPDC-109 and mutants. Absorption due to haemoglobin released upon incubation of 0.8% erythrocyte suspension with 100 µg protein for 60 minutes was monitored at 415 nm.

Confocal imaging with the W47A mutant showed some change in morphology after 30 minutes of incubation **(Fig. 9C)**; upon increasing the incubation period to 1 h, it was observed that the erythrocyte membrane was completely disrupted **(Fig. 9D)**. However W93A and W106A mutants had no effect on the morphology of erythrocytes even after incubation for 1h **(Fig. 9E and 9F)**. This was further confirmed by the erythrocyte lysis assay, which showed that only the W47A mutant exhibited the ability to lyse erythrocytes, although with a significantly reduced efficiency (10~15% as compared to rPDC-109). W93A and W106A mutants could not lyse the erythrocytes even after 1 hr of incubation **(Fig. 9G)**.

4.8 Glycosylation of PDC-109 increases its CLA and tryptophan residues at position 47, 93 and 106 are crucial for it

In order to understand the role of glycosylation and ligand-binding residues in the CLA of PDC-109, heat-induced aggregation of ADH was used as a model system. Thermal aggregation was studied at different weight ratios of PDC-109 and the target protein. It was found that both glycosylated, wild type PDC-109 and non-glycosylated recombinant PDC-109 were effective in preventing aggregation of the target protein (**Fig. 10A**). The effect of glycosylation of PDC-109 on the prevention of heat-induced aggregation of target protein was further studied by atomic force microscopy imaging. Under similar thermal stress condition, target protein alone and target protein along with rPDC-109 formed very large aggregates **(Fig. 10D and 10F)**. On the other hand, target protein incubated with WT PDC-109 formed aggregates which were intermediate in size **(Fig. 10E)**. A control experiment was performed with target protein alone, which was incubated at 4 °C. The AFM image of control experiment showed equally distributed tiny particles of the target protein **(Fig. 10C)**. The above results demonstrate that wild-type protein exhibits better CLA than recombinant PDC-109, indicating that absence of glycosylation reduces the CLA of PDC-109.

The ability of the mutants to prevent the thermal aggregation of ADH was studied at a target protein to chaperone ratio of 1:1(w/w) **(Fig. 11).** It was found that W47A was able to protect the target protein; however, the chaperone-like activity of the W47A mutant decreased to about half of that exhibited by rPDC-109 with the wild type sequence. Mutations in FnII domain 2 viz., W93A and W106A resulted in a complete loss in the ability of PDC-109 to protect the target protein from thermal aggregation (**Fig. 11**). In fact, in the presence of these mutants, higher aggregation was observed than for ADH alone. In order to find out whether the mutant proteins self-aggregate under the conditions of the chaperone assay, we have monitored the change in light scattering of these proteins alone, in the absence of the target protein. No increase in light scattering was observed when the W93A or the W106A mutants were incubated at 48 °C, indicating that they do not self-aggregate **(Fig. 12).**

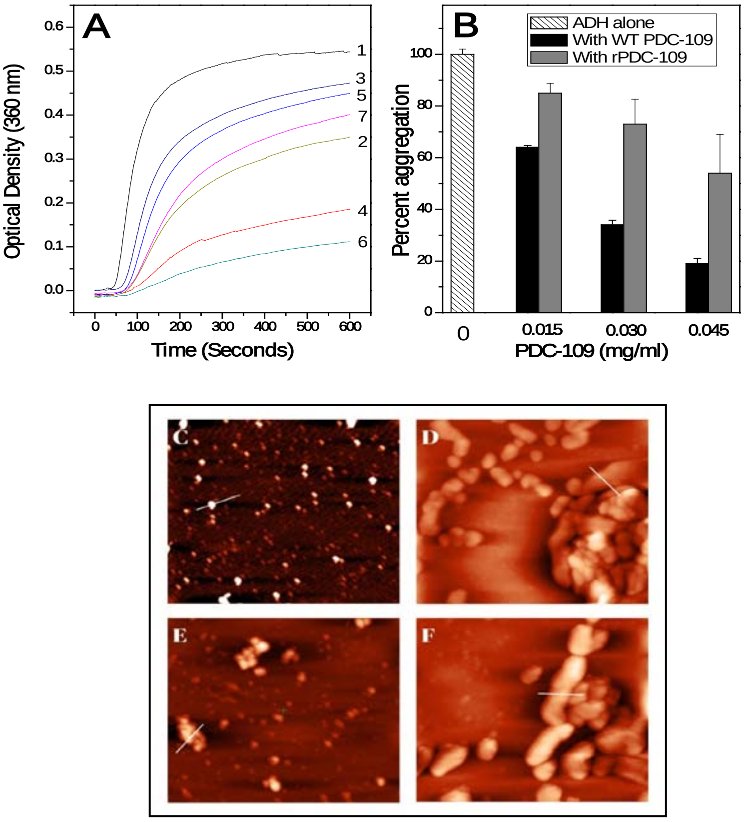


Figure 10. (A) Aggregation assay of ADH. ADH concentration was 0.1 mg/ml for all samples and all samples were incubated at 48°C. *Curve*1- ADH alone; *curves* 2 and 3, ADH incubated with 0.015mg/ml of WT PDC-109 and rPDC-109, respectively; c*urve* 4 and 5, ADH incubated with 0.03 mg/ml WT PDC-109 and rPDC-109, respectively; *curves* 6 and 7, ADH incubated with 0.045 mg/ml WT PDC-109 and rPDC-109, respectively. (**B**). Bar diagram representation of date shown in (**A**), identification of various samples is indicated in the figure. Error bars are too small for some of the samples and hence are not seen. (**C-F**) AFM images showing heat induced aggregation of ADH. Protein concentration was 0.04 mg/ml in all samples. Images shown correspond to ADH that was incubated at 4°C (**C**), at 48°C for 30 minutes (**D**), with 0.02 mg/ml WT PDC-109 at 48°C for 30 minutes, with 0.02 mg/ml rPDC-109 at 48°C for 30 minutes. All images have the size of 5×5 µM.

The higher aggregation observed in the presence of target protein, therefore, appears to arise from the co-aggregation of mutant PDC-109 and target protein.

These observations suggest that the conserved tryptophan residues in the FnII domains are important both for the ligand-binding as well as the chaperone-like activity of PDC-109.

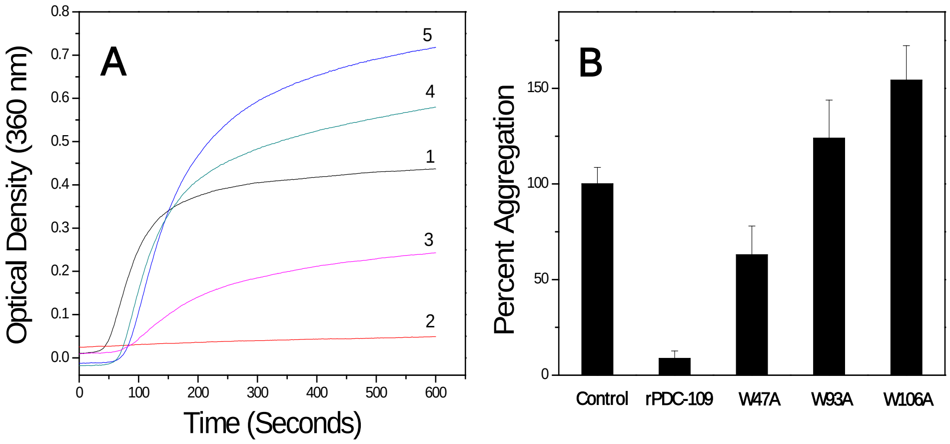


Figure 11. ADH Aggregation assay. (A) Thermal aggregation of alcohol dehydrogenase was investigated at 48°C in the presence and absence of rPDC-109 and mutants (W47A, W93A and W106A). In all experiments, final concentrations of ADH and rPDC-109 (or mutants) was 0.1 mg/ml and 0.15 mg/ml, respectively. *Curve 1*, ADH alone; *Curve 2*, ADH in presence of rPDC-109; *curves 3*, *4* and *5*, ADH in presence of W47A, W93A and W106A, respectively. (**B**) Bar diagram representing percent aggregation of ADH alone (control) and in presence of rPDC-109 and different mutants.

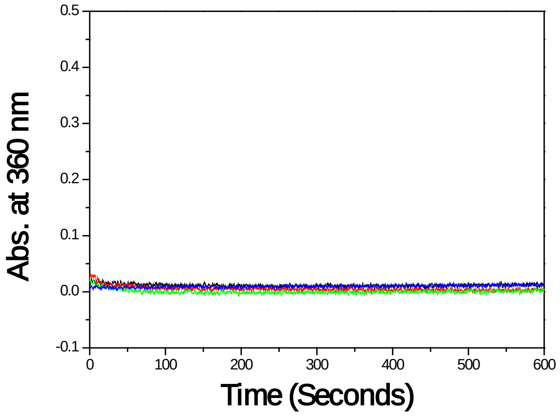


Figure 12: Effect of temperature on self-aggregation of rPDC-109 and mutants W47A, W93A and W106A. See text for details.

4.9 Mutation in Trp residues at 47, 93 and 106 increases the surface hydrophobicity Surface hydrophobicity of all the mutant was compared with rPDC-109 using bis-ANS probe. The results obtained show that mutation of the conserved Trp residue leads to an increase in the surface hydrophobicity for all three mutants **(Fig. 13)**.

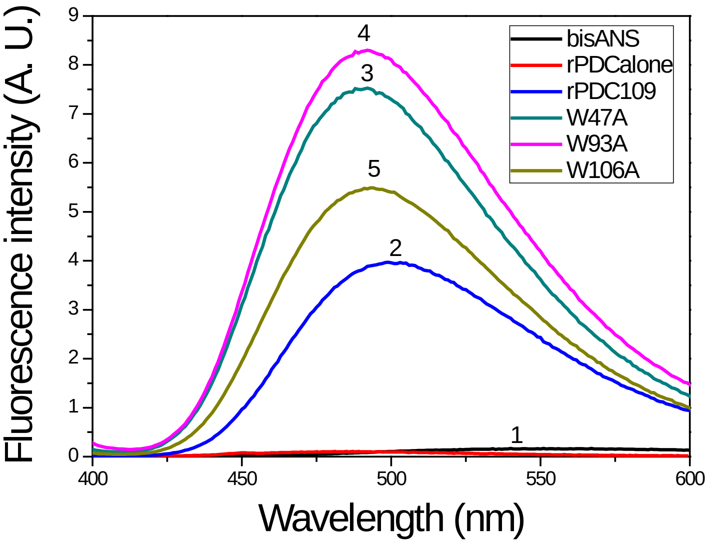


Figure 13. bis-ANS-binding studies. bis-ANS, and the protein were mixed to give a final concentration of 10 µM each. The samples were excited at 390 nm and emission spectra were recorded with the excitation and emission band passes set at 2 and 3 nm, respectively. *Spectrum 1,* bis-ANS probe in buffer; *spectrum 2,* bis-ANS + rPDC-109; *spectra 3, 4* and *5,* bis-ANS with mutants W47A, W93A and W106A, respectively. Dotted line corresponds to rPDC-109 in buffer without probe.

4.10 Trp residues at 47, 93 and 106 are important for oligomerization

PDC-109 is a polydisperse, oligomeric protein; interaction with PrC decreases the size of the oligomers and also reduces its CLA (Sankhala and Swamy, 2010; Gasset *et al.*, 1997). In order to investigate the effect of glycosylation and mutation of Trp residues on the oligomeric state of PDC-109, we performed glutaraldehyde cross-linking on wild type PDC-109, rPDC-109 and the tryptophan mutants **(Fig. 14)**. Both wild type and recombinant PDC-109 exhibit a prominent band corresponding to a monomer, but also exhibit bands corresponding to dimer, trimer and higher oligomers. On the other hand, W47A, W93A and W106A show only a single band corresponding to the monomer, clearly indicating that the conserved Trp residues are important for maintaining polydispersity of the protein.

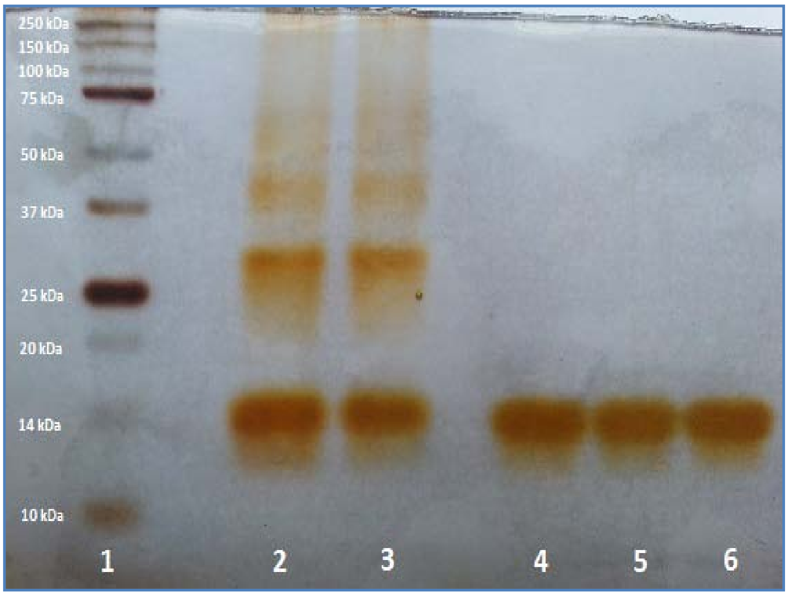


Figure 14. A 15% SDS polyacrylamide gel of glutaraldehyde cross-linked proteins. (lane 1) Marker proteins, (lane 2) WT PDC-109, (lane 3) rPDC-109, (lane 4) W47A, (lane 5) W93A and (lane 6) W106A.

5. Discussion

In the present study, we have investigated the effect of glycosylation on the structure, chaperone-like activity and lipid-binding ability of PDC-109. Recombinant PDC-109 was expressed in *E. coli* in inclusion bodies and could be refolded from its guanidine hydrochloride unfolded and DTT-reduced state using the GSH/GSSG redox system. Redox systems such as GSH/GSSG and cysteine/cystine continuously modulate inter- or intra-chain disulphide bonds until a perfectly refolded protein structure is achieved (Ahmed *et al.*, 1975; Creighton 1992; Raman *et al.*, 1996).

Near- and far-UV CD spectra of rPDC-109 are similar to those of wild type PDC-109 isolated from bovine seminal plasma, showing that the recombinant protein has refolded to its native state. Glutaraldehyde cross-linking studies showed that rPDC formed higher oligomers like wild type PDC-109. Results of DSC studies demonstrate that glycosylation does not affect the oligomerization or polydispersity of PDC-109. However, the unfolding temperature of the monomer is about 2 °C lower than that reported for WT PDC-109 (Gasset *et al.*, 1997; Damai *et al.*, 2015). This observation suggests that the slightly lower thermal stability of recombinant PDC-109 is probably due to the absence of glycosylation since glycosylation has been reported to enhance the stability of a number of proteins (Mitra *et al.*, 2006; Chen *et al.*, 2014).

PDC-109 is a choline phospholipid-binding protein. Binding of phosphorylcholine and choline-containing phospholipids such as Lyso-PC and DMPC to it result in a significant blue shift in the fluorescence emission λ_{max} of the protein accompanied by a large increase in the fluorescence intensity (Müller *et al.*, 1998; Anbazhagan *et al.*, 2008). Recombinant PDC-109 also binds to PrC, lysoPC and DMPC with similar (or slightly stronger) affinity as compared to wild type PDC-109. PDC-109 is known to bind to the outer surface of sperm plasma membrane, where choline phospholipids such as phosphatidylcholine and sphingomyelin are highly abundant (Lenzi *et al.*, 1996; Tannert *et al.*, 2007). The outer leaflet of the erythrocyte membranes is also largely composed of such phospholipids (Zwaal and Schroit, 1997). Therefore, erythrocytes provide an excellent model system to investigate the interaction of PDC-109 with cell membranes. In previous work we have shown that binding of PDC-109 to human erythrocytes results in a disruption of the plasma membrane and release of hemoglobin into the solution, which was dependent on the protein concentration and incubation time (Damai *et al.*, 2010). Interestingly, this study shows that the ability of recombinant PDC-109 to induce lipid efflux, as monitored by erythrocyte lysis, is higher compared to that of wild type PDC-109. In previous work, we observed that upon binding to model membranes containing choline phospholipids, PDC-109 penetrates into the lipid bilayer and removes lipids from the membrane (Ramakrishnan *et al.*, 2001). It is possible that the glycan moiety of wild type PDC-109 causes steric hindrance, thereby affecting its ability to insert into the lipid bilayer. Lack of glycosylation would then facilitate insertion of the protein into the lipid bilayer, making it more efficient in inducing lipid efflux.

Proteins in the extracellular space are exposed to challenging conditions such as oxidising environment and high calcium concentrations (Hebert and Molinari, 2007). Several diseases involve the deposition of proteins in the extracellular space (Wyatt *et al.*, 2013). Some proteins in the extracellular fluids such as clusterin and haptoglobin may function as extracellular chaperones, preventing aggregation of proteins and clearing them from the extracellular space (Humphreys *et al.*, 1999, Wyatt *et al.*, 2011, Sultan *et al.*, 2013). In the previous work, we demonstrated that the major bovine seminal plasma protein, PDC-109 and its equine homologue, HSP 1/2 display the ability to prevent amorphous as well as amyloid aggregation of various target proteins (Sankhala and Swamy, 2010, Sankhala *et al.*, 2012). However, the role of glycosylation in the ligand-binding and chaperone-like activity of PDC-109 is not known. The present results demonstrate that wild-type PDC-109 exhibits better CLA than rPDC-109, indicating that glycosylation plays a positive modulatory role in the CLA. Interestingly, deglycosylation of another extracellular chaperone present in the seminal plasma, clusterin, had no effect on its secondary structural content, but led to slightly increased surface hydrophobicity and slightly higher chaperone–like activity than the wild type, glycosylated protein in preventing thermally induced aggregation of citrate synthase at pH 7.5, though no significant difference was observed at pH 6.0 between the glycosylated and deglycosylated forms (Stewart *et al.*, 2007).

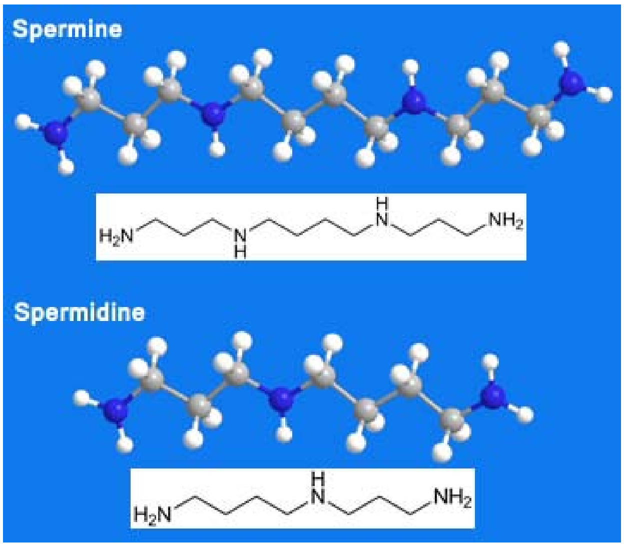
Another aspect of PDC-109 that has been investigated in this study relates to the role of the conserved core tryptophan residues in its two FnII domains. We have mutated these conserved core tryptophan residues to alanine in order to understand their role in structure, lipid-binding ability and chaperone-like activity. Far- and near-UV CD spectra, as well as intrinsic fluorescence spectra of the W47A, W93A and W106A mutant proteins refolded from their denatured/reduced states are not significantly different from those of the wild type PDC-109. The mutant proteins show very little or no lipid-binding ability as monitored by fluorescence studies of PrC binding and erythrocyte lysis assay, clearly indicating that the tryptophan residues in both domain I (W47) and domain II (W93 and W106) are important for ligand-binding. These results are of significant interest, as under *in vivo* conditions, PDC-109 interacts with sperm plasma membranes and facilitates the capacitation of spermatozoa, which is an obligatory step for successful fertilization. Beside this, since core tryptophan residues are conserved across all FnII domains as well as in kringle domains, similar studies could help understand protein-ligand interactions of other proteins containing FnII domains and the significance of such interactions. For example, previous studies show that many pathogenic bacteria and viruses interact with their surface protein with the FnII domain of fibronectin and that such binding is necessary for their life cycle (Petro *et al.*, 1981; Atkin *et al.*, 2010; Larson *et al.*, 2013). Comparison of solution structure of FnII–domain of fibronectin and domain II of PDC-109 shows similar solvent-exposed orientation of core tryptophan (Constantine *et al.*, 1992; Pickford *et al.*, 1996). Similar studies with FnII domains of other proteins can therefore prove to be of potential medical application.

PDC-109 is a polydisperse, oligomeric protein (Gasset *et al.*, 1997); interaction with PrC decreases the size of the oligomers and also reduces its chaperone-like activity (Gasset *et al.*, 1997; Sankhala and Swamy, 2010). The present study shows that mutation of the conserved core tryptophan residues in the two FnII domains of PDC-109 results in decreased oligomer formation as confirmed by glutaraldehyde cross-linking, which shows only a single band for mutants corresponding to monomeric form whereas higher oligomers were seen for WT and rPDC-109 (Figure 11). While one of the mutants (W47A) exhibits significantly reduced chaperone-like activity, the other mutants (W93A and W106A) do not show any chaperone-like activity and in fact co-aggregate with the target protein. These results suggest that the tryptophan residues at positions 93 and 106 are important for the stability of PDC-109. Substitution at these positions with alanine probably renders the protein less stable, leading to their co-aggregation with the target protein in the aggregation assay. However, our results show that these proteins do not self-aggregate in the absence of the target proteins under the conditions used in the chaperone assay (Figure 12). Interestingly, the mutant proteins exhibit increased bis-ANS binding (Figure 13), indicating that these proteins have significantly higher surface hydrophobicity compared to the wild type protein. Increased surface hydrophobicity is expected to increase the CLA of chaperones. However, instances of decreased chaperone-like activity and co-aggregation with the target protein have been reported in some cases (Singh *et al.*, 2006; Koteiche and Mchaourab, 2006). The present results show that W93A and W106A mutations in PDC-109 do not affect the ability of the mutant proteins to bind the target protein (ADH), but probably affect the propensity of the complex to aggregate. Taken together, our results demonstrate that both surface hydrophobicity and oligomeric status of PDC-109 are important determinants of its chaperone-like activity.

In summary, in the present study we have cloned and expressed the major bovine seminal plasma protein PDC-109 in *E. coli*. DSC studies showed that the thermal stability of rPDC-109, which was devoid of covalently bound carbohydrate was similar to that of the wild-type protein. However, rPDC-109 exhibited a higher ability to lyse erythrocytes, but had a lower chaperone-like activity. Mutational analysis reveals that a core tryptophan residue in each FnII domain of PDC-109, which is highly conserved across all proteins with FnII domains, is crucial for both ligand-binding and chaperone-like activities of this protein.

Chapter 3

Spermine and Spermidine Act as Chemical Chaperones, and Enhance the Chaperone-Like Activity and Lipid Binding Ability of PDC-109



Structure of Spermine and Spermidine. Reproduced from: Molecule of the week, American Chemical Society, February 13, 2012.

1. Abstract

Presence of polycationic molecules like spermine (Spm) and spermidine (Spd) in the seminal plasma of many mammalian species is known for long. Previous studies have shown that both PDC-109 and spermine bind to phosphatidylcholine on the sperm plasma membrane. In this study the effect of Spm/Spd on the lipid efflux inducing ability and chaperone like activity of PDC-109 was investigated by utilizing various biochemical and biophysical methods. The results obtained show that in the presence of Spm/Spd lipid efflux induced by PDC-109 increases, which is a crucial step before fertilization. In the presence of Spm/Spd, PDC-109 provides better protection to many target proteins from thermal stress condition and helps them to retain the activity and prevents them from self-aggregation, as compared to in presence of PDC-109 alone. When Spm or Spd was used in the absence of PDC-109, it was observed that they themselves were able to provide protection to target protein in a concentration dependent manner. Analysis of results shows that when PDC-109 and Spm/Spd are used together, the CLA observed is more than that expected for a simple additive effect, suggesting that PDC-109 and the polyamines act in a synergistic fashion, with spermine being more effective than spermidine.

2. Introduction

As described in Chapter 1, in mammals, at the time of ejaculation, epididymal sperm comes in contact with seminal plasma and their surface becomes coated with various factors present in the seminal plasma. These factors play many crucial roles after deposition of sperm in the female reproductive tract, both prior to and at the time of fertilization. In bovine, binding of PDC-109 to the sperm surface and its role in removal of lipid molecules from the plasma membrane of sperm during acrosome reaction is well established.

The natural polyamines spermine (Spm) and spermidine (Spd) are small and flexible polycationic alkylamines, which are ubiquitous in their presence. They play an important role, in both normal and disease-altered tissues. Extensive studies on polyamines have shown that they play important roles in many cellular processes, including regulation of gene expression, functioning of ion channels, cell migration, resistance from stress and infectious diseases, nucleic acid packaging, DNA replication, apoptosis, transcription and translation (Williams, 1997; Ruan *et al.*, 1994). Their importance in these functions has been studied extensively and has been described in detail in recent reviews (Igarashi and Kashiwagi, 2010; Nowotarski *et al.*, 2013; Pegg, 2014). Presence of Spm in human seminal plasma was discovered more than three centuries ago by Leeuwenhoek, where it is present at concentrations ranging from 2.5 to 17.5 mM. Further studies reported that Spm is present in millimolar concentration in the seminal plasma of many other mammalian species as well, which is much higher than its presence in other body fluids (Atmar *et al.*, 1981; Rubinstein *et al.* 1995). Studies with bovine spermatozoa show dual effect of Spm; at low concentration it promotes acrosomal exocytosis and at high concentration it inhibits it (Rubinstein *et al.*, 1995). Binding studies of Spm with ram spermatozoa show that binding and release rates are faster compared to somatic cells. Further, release of Spm from membrane is facilitated by heparin, which is a major component of the secretions of the female reproductive tract (Rubinstein *et al.* 1991).

As Spm and PDC-109 both bind to sperm plasma membrane, we considered it would be of interest to investigate the effect of Spm and its precursor Spd on lipid efflux ability of PDC-109. Our results from interaction of PDC-109 with erythrocyte membrane and DMPC model membrane show that the lipid efflux ability of PDC-109 increases in the presence of Spm/Spd.

In the present study we also explored the effect of Spm/Spd on the chaperone like activity of PDC-109, as it is known to share functional characteristics of extracellular chaperones and protect a variety of target proteins from various stress conditions (Sankhala and Swamy, 2010). Studies from other groups have shown differential effect of polyamines on protein aggregation. One study shows that Spm has a protective effect on lysozyme under conditions of thermal denaturation. However, these studies reported the use of 50-100 mM concentration of Spm (Shiraki *et al.*, 2003), which is several fold higher than its physiological concentration. On the other hand, some studies have shown that presence of 10 μM to 1 mM Spm or Spd increases the aggregation and fibrillization of α-synuclein and amyloid-beta (Aβ) peptide by shortening the lag and transition time of fibril formation (Antony *et al.*, 2003; Luo *et al.*, 2013). In the present study we had used physiological concentration of Spm/Spd to observe its effect on heat induced denaturation and aggregation of various target proteins such as glucose-6-phosphate dehydrogenase (G6PD), lactate dehydrogenase (LDH), carbonic anhydrase (CA) and aldolase. The results show that at these concentrations Spm and Spd are effective in preventing heat induced aggregation and inactivation of target proteins and when both PDC-109 and Spm/Spd are used together, the CLA observed is more than that expected for a simple additive effect, i.e., they act in a synergistic fashion.

3. Materials and methods

3.1 Materials

Spermine, spermidine, glucose-6-phosphate dehydrogenase (G6PD) from Bakers yeast, glucose-6-phosphate (G6P), sodium L-lactate and carbonic anhydrase (CA) were purchased from Sigma (St. Louis, MO, USA). Lactate dehydrogense (LDH), aldolase, nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide free acid (NAD^+), Tris base and other chemicals were purchased from local suppliers and were of the highest purity available. PDC-109 was purified as described earlier (Ramakrishnan *et al.*, 2001). Spermine or spermidine dissolved in TBS-I buffer and pH adjusted to 7.5 using 1 N HCl, were used throughout the work.

3.2. Erythrocyte lysis assay

The effect of Spm/Spd on the ability of PDC-109 to disrupt the plasma membrane of human erythrocytes was investigated by monitoring the release of haemoglobin as described earlier (Damai *et al.*, 2010). PDC-109 (100 µg) was pre-incubated with Spm/Spd for about 2 minutes and the volume was then adjusted to 800 µl with TBS-I buffer. The reaction was started with the addition of 200 µl of 4% RBC suspension, making the final volume 1.0 ml. The whole mixture was then incubated for 1 hour at room temperature, followed by centrifugation of the samples at 3000 rpm for 10 minutes. The supernatant was collected and its optical density was measured at 415 nm (which corresponds to the absorption by the haem moiety of haemoglobin). Control experiments were performed in the same way in the absence of PDC-109.

3.3 Confocal microscopy

The effect of spermine on PDC-109-induced membrane disruption and modulation of erythrocyte morphology was further investigated by confocal microscopy. To monitor the effect of Spm on lipid efflux ability of PDC-109, a 0.1% erythrocyte suspension was incubated with 0.25 mg/ml PDC-109 and 2.5 mM Spm. Aliquots were drawn from the the erythrocyte suspension at regular intervals and directly spotted on a clean glass slide and then transferred to the confocal stage for imaging. Imaging was done using a Leica TCS SP2 confocal microscope (Heidelberg, Germany) in the transmission mode. In control experiments imaging was done with 0.1 % erythrocyte solution incubated with Spm alone.

3.4 Preparation of DMPC model membrane

About 1 mg of DMPC was taken in a glass test tube and dissolved in 1 ml of chloroform-methanol (1:1, v/v) mixture. The solvent was evaporated by passing a gentle stream of nitrogen gas over the sample and then kept under vacuum desiccation for 3-4 h to remove any residual traces of the solvent. A thin film formed on the glass surface was hydrated with TBS-I buffer to make the final concentration 500 µM and the sample was then sonicated for 1h in a bath sonicator and stored at 4 °C until use.

3.5 Enzyme activity assay

To investigate the effect of Spm/Spd on the chaperone-like activity of PDC-109, enzyme assays were performed for the activity of G6PD and LDH under thermal stress using spectrophotometric methods as described previously (Sankhala and

Swamy, 2010; Kumar *et al.*, 2005). In the G6PD activity assay G6P is oxidized into 6-phospho-D-gluconate by the action of G6PD, which simultaneously uses NADP as a cofactor and converts it into NADPH. The reaction was initiated by the addition of NADP to a mixture containing G6PD (0.25 µM), NADP (0.1 mM), G6P (5 mM) and 12 mM each of $MgCl_2$ and KCl, and increase in the absorbance at 340 nm by NADP was monitored by recording the absorbance at 340 nm as a function of time in an Agilent Technologies Cary 100 double beam UV/Visible spectrophotometer, equipped with a Peltier accessory, which was also used for all other spectrophotometric measurements. To investigate the effect of Spm on the CLA of PDC-109 against thermal denaturation of G6PD, the sample was incubated with 0, 2.5 and 5 mM Spm along with 100 µg of PDC-109 for about 2 minutes and then transferred to a water bath which was stabilized at $45^{o}C$ for 30 min. A control experiment was performed with Spm and PDC-109 alone. Relative activities of various treated samples were normalized with respect to that of the native enzyme.

In the LDH activity assay, lactate is converted to pyruvate by LDH with simultaneous reduction of NAD^+ to NADH. The assay mixture contained LDH (0.7 µM), NAD^+ (0.2 mM) and sodium lactate (100 mM) in TBS-1 buffer. The reaction was initiated by adding the enzyme and increase in absorbance at 340 nm due to the reduction of NAD^+ was monitored at room temperature. To evaluate the effect of Spm on the CLA of PDC-109, similar experimental procedure was followed as described above for the G6PD activity assay. To study the effect of Spd on the CLA of PDC-109, a similar experiment was performed with spermidine also.

3.6 Aggregation assay

To investigate the effect of Spm/Spd on the ability of PDC-109 to prevent heat-induced aggregation of target proteins, experiments were performed with LDH and aldolase as described previously (Horwitz *et.al.*,1992; Sankhala and Swamy, 2010). A 0.1 mg/ml sample of LDH was kept in a spectrophotometer cell holder which was thermally stabilized at 48 °C and aggregation was monitored by recording light scattering at 360 nm as a function of time. A fixed concentration of PDC-109 (0.05 mg/ml) in TBS-I was pre-incubated with Spm (2.5 mM to 10 mM range) for 2 min at room temperature and the target protein (LDH or aldolase) was then added. After this the experiments were performed as described above. Aggregation profile for the native enzyme was taken as 100% and percent aggregation of other samples were calculated with respect to that of the native enzyme. Aggregation assays performed in the presence of different concentrations of Spm (2.5 mM to 10 mM range) and PDC-109 alone were treated as control experiments. Similar aggregation inhibition assay of aldolase was performed with both Spm and Spd.

3.7 Atomic force microscopy (AFM)

The effect of Spm on the ability of PDC-109 to disrupt DMPC model membranes, was investigated by AFM as described earlier (Damai *et. al.*, 2010). Multilamellar vesicles containing 500 µM DMPC were prepared from which samples containing 300 µM DMPC and 0.5 mg/ml PDC-109 with or without 2.5 mM Spm were made by adding appropriate aliquots of the protein and the polyamine. In a control experiment 300 µM DMPC solution was prepared with 2.5 mM Spm but without PDC-109. Immediately after sample preparation about 50-60 µl of sample was pipetted on to a freshly cleaved mica sheet (1cm × 1 cm). The sample was allowed to dry completely at room temperature and then rinsed with HPLC grade water, dried and transferred to the AFM stage.

The effect of Spm and PDC-109 on the thermal aggregation of carbonic anhydrase was investigated by a procedure similar to that described earlier (Sankhala and Swamy, 2010). A 40 µg/ml sample of CA was incubated for 2 minutes with 20 µg/ml of PDC-109 and the volume was adjusted to 500 µl. To investigate the effect of Spm 40 µg of CA was incubated with 2.5 mM of spermine. A control experiment was performed with 40 µg of CA in the absence of PDC-109 and Spm. All the samples were kept at 45 °C in a water bath for 20 minutes and then transferred to room temperature. A 50-60 µl aliquot of the sample solution was carefully deposited on a freshly cleaved mica sheet (1 cm × 1 cm) and allowed to dry for 20-30 min at room temperature, rinsed with HPLC grade water, dried, and transferred to the AFM stage.

Imaging was performed in semicontact mode using a SOLVER PRO-M atomic force microscope (NT-MDT, Moscow, Russia), which was equipped with a 10.0 µm bottom scanner. NSG10 cantilevers with Au reflective coating and a nominal spring constant of 11.8 N/m were used for the scanning. Force was adjusted continuously for the lowest possible value by adjusting the set point and feedback gain during imaging. Images were analyzed using NOVA software, supplied by NTMDT.

4. Results

4.1 Spm/Spd increase lipid efflux ability of PDC-109 from erythrocyte plasma membrane

Incubation of 0.8% erythrocyte suspension for one hour in the presence of PDC-109 led to a disruption of the erythrocyte plasma membrane, resulting in the release of haemoglobin into the solution, which was determined spectrophotometrically (Fig. 1A). In the presence of 100 µg/ml of PDC-109 optical density at 415 nm was observed to be 0.24. Addition of spermine at 2.5 to 10 mM resultant concentration in the reaction mixture resulted in increasing extents of lysis as assessed by increase in the optical density with increase in the concentration of spermine (Fig. 1A). Addition of Spm to the erythrocyte solution in the absence of PDC-109 resulted in negligible changes in the optical density indicating that Spm alone does not affect the erythrocyte membrane integrity. Similar experiments with Spd indicated that the effect of spermidine on PDC-109 induced erythrocyte lysis was less as compared to spermine (Fig. 1B).

The ability of spermine to enhance PDC-109-induced erythrocyte membrane lysis was further confirmed by confocal microscopy. Monitoring of erythrocyte morphology after incubation with PDC-109 at regular intervals showed that erythrocytes started losing their integrity and were fragmented, yielding small vesicular structures at about 30 minutes (Fig. 1D). Under similar condition, presence of 2.5 mM Spm caused deformation of cell morphology and lysis in just 10 minutes (Fig. 1E). Increasing the incubation time upto 30 minutes resulted in complete lysis of the cells (Fig. 1F). Erythrocytes incubated with 2.5 mM spermine for 2 h did not show any deformity in their morphology (Fig, 1C).

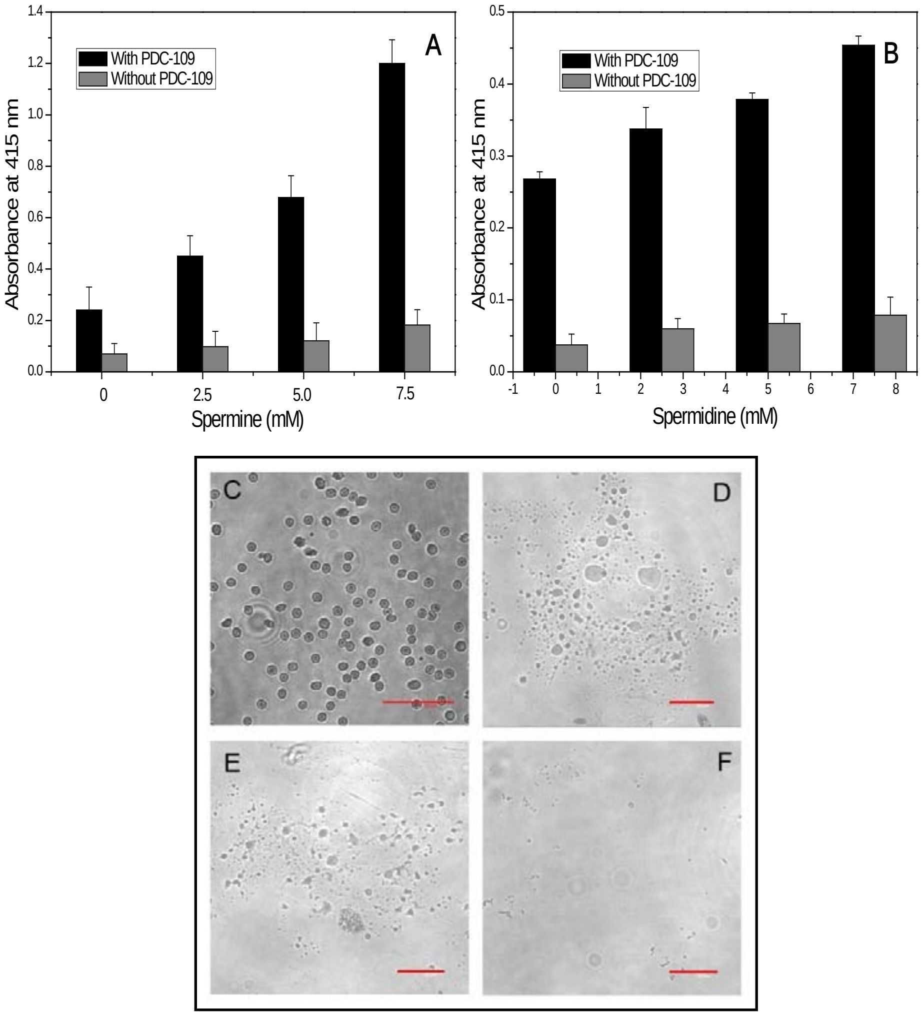


Fig. 1. Effect of Spm/Spd on membrane destabilization ability of PDC-109. Optical density at 415 nm after incubation of 0.8 % human erythrocyte with 100 µg/ml PDC-109 for 1 h at room temperature in presence of different concentration (0, 2.5, 5 and 7.5 mM) of (**A**) Spm and (**B**) Spd. Confocal image of 0.1 % human erythrocyte in TBS-1 buffer after incubation with (**C**) 2.5 mM Spm for 1 h, (**D**) 0.25 mg/ml PDC-109 for 30 minutes, (**E**) 0.25 mg/ml PDC-109 + 2.5 mM Spm for 10 minutes, and (**F**) 0.25 mg/ml PDC-109 + 2.5 mM Spm for 30 minutes. Scale bar, 50 µm.

4.2 PDC-109 shows higher ability to disrupt DMPC-model membrane in the presence of Spm

DMPC model membranes incubated with 5 mM Spm showed well defined multilamellar structure as observed by AFM (Figure 2A). When DMPC vesicles were incubated with 0.5 mg/ml PDC-109, the multilamellar structure was disrupted and small membrane fragments were observed (Figure 2B). Under similar condition, addition of 5 mM Spm to the reaction mixture resulted in complete disruprtion of DMPC vesicles, and very small fragment were observed (Figure 2C). Thus, presence of Spm resulted in an enhancement of PDC-109 activity, which is in agreement with the results obtained from their interaction with erythrocyte plasma membrane as described above.

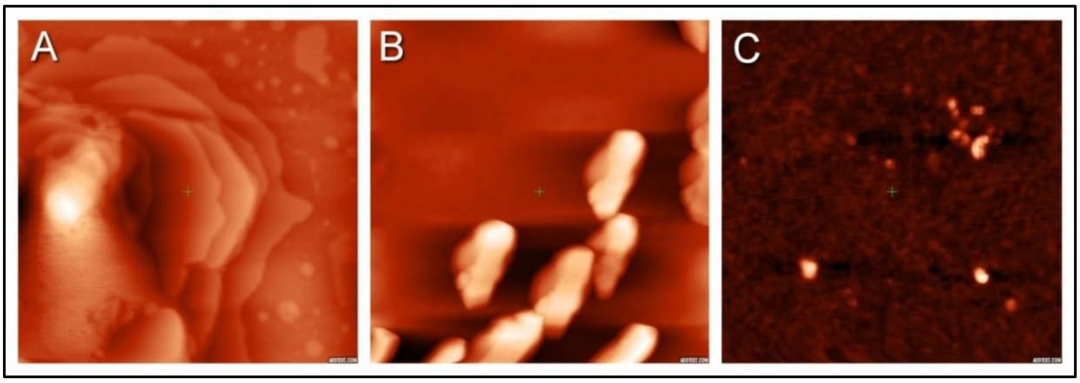


Fig. 2. AFM studies on the effect of spermine on DMPC model membrane-PDC-109 interaction. Images are shown for DMPC model membranes (**A**) in presence of 5 mM spermine, (**B**) in presence of 0.5 mg/ml PDC-109, and (**C**) in presence of 0.5 mg/ml PDC-109 and 5 mM spermine. All images are 5 × 5 µm in size. See text for details.

4.3 Polyamines Spm and Spd protect the G6PD and LDH from thermal denaturation

Incubation of G6PD at 45 ^{o}C for 10 minutes resulted in a drastic reduction of its activity with only about 19% activity being seen (Fig. 3A, curve 2) with respect to its activity observed at room temperature (Fig. 3A, curve 1). On the other hand 30.4%, 40.7% and 64.3% activity was retained when the enzyme was subjected to a similar treatment in the presence of spermine (2.5 mM, 5 mM) and PDC-109 (0.05 mg/ml), respectively (Fig.3A curves 3, 4 and 5). In the presence of 2.5 and 5 mM of spermine and 0.05 mg/ml PDC-109 the activities retained were 77.49% and 89.4% (Fig. 3A, curves 6 and 7). Percent activities of different samples are shown as a bar diagram in Fig. 3B and also are summarized in Table 1.

Incubation of LDH at 45 ^{o}C for 10 min resulted in only12% of its activity being retained (Fig. 3C, curve 2) as compared to its activity measured at room temperature (Fig. 3C, curve 1), whereas 26.8%, 37.5% and 53.6% activity were retained when the enzyme was subjected to similar treatment (incubation at 45 °C for 10 min) in the presence of Spm (concentration 2.5 mM, 5 mM) and PDC-109 (3.5 µM), respectively (Fig.3C, curves 3,4 and 5). When Spm at the same concentrations (2.5 mM and 5 mM, respectively) was preincubated with PDC-109 (0.05 mg/ml) the activities retained increased to 73.2% and 93% (Fig.3C, curves 6 and 7).

To compare the effect of Spm and Spd on the CLA of PDC-109, experiments similar to those described above were carried out with Spd (Fig. 3E). It was observed that in the presence of 2.5 mM and 5 mM Spd 22.2% and 37% activity were retained (Fig. 3E, curve 3 and 4). Preincubation of Spd (2.5 mM and 5 mM) and PDC-109 (0.05 mg/ml) resulted in the retention of 59.2% and 78% activity, respectively (Fig.3E, curves 6 and 7). Thus, it can be inferred that for similar concentration of Spd alone and for Spd preincubated with PDC-109, the effect was less compared to that of Spm in protecting the heat treated enzyme. Results for effect of Spm/Spd on LDH activity are summarized in Table 2.

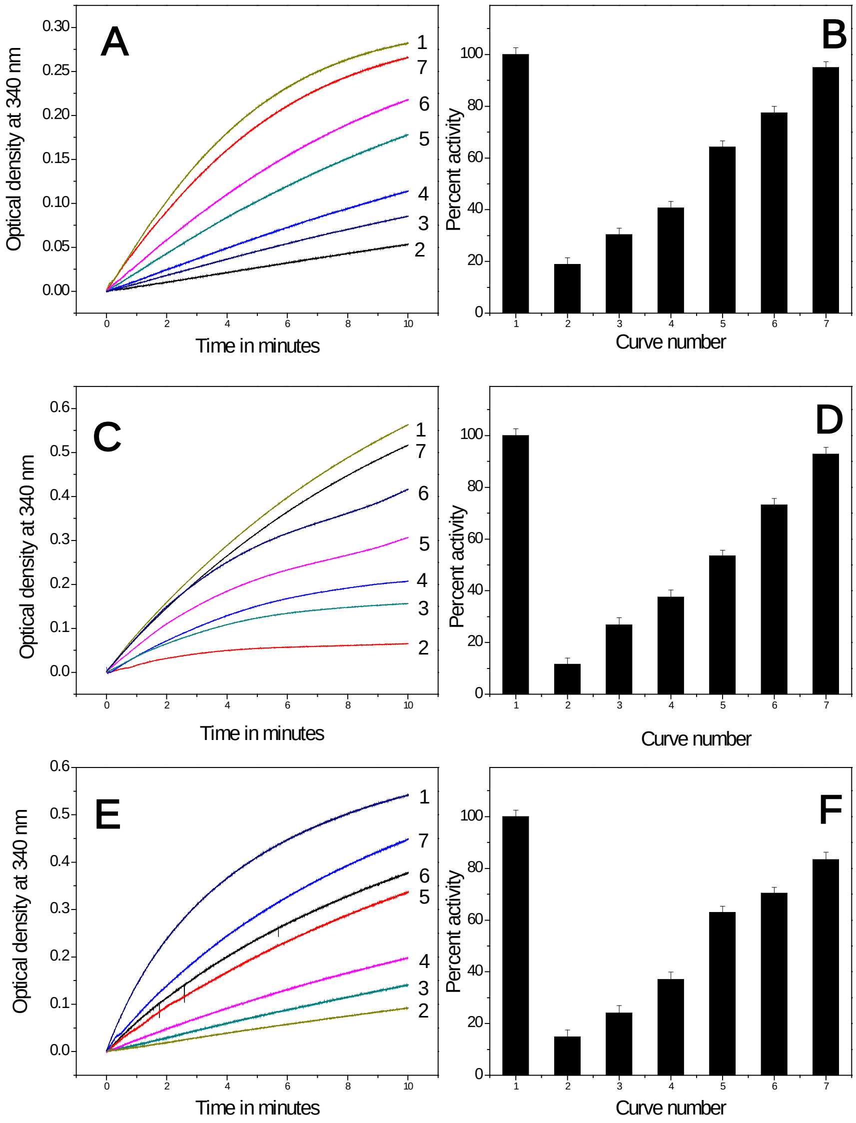


Fig. 3. Effect of Spm/Spd on CLA of PDC-109 against heat denatured enzymes (**A**) PDC-109 and spermine assisted protection of G6PD. Activity of the enzyme at room temperature (*curve 1*) and after incubation at 45°C for 30 minutes: in buffer alone (*curve*

2), with 2.5 mM Spm (*curve 3*), with 5 mM Spm (*curve 4*), with 100 µg/ml PDC-109 (*curve 5*), with 100 µg/ml PDC-109+2.5 mM Spm (*curve 6*) and µg/ml PDC-109+5 mM Spm (*curve 7*). (**B**) Bar diagram representation of the activity of G6PD of panel A. (**C**) PDC 109 and Spm assisted protection of LDH. Activity of the enzyme at room temperature (*curve* 1) and after incubation at 45°C for 45 minutes: in buffer alone (*curve 2*), with 2.5 mM Spm (*curve 3*), 5 mM Spm (*curve 4*), 100 µg PDC-109 (*curve 5*), PDC-109+2.5 mM Spm (*curve 6*), PDC-109+5 mM Spm (*curve 7*). (**D**) Bar diagram representing the percent activity of LDH of panel C. (**E**) PDC 109 and Spd assisted protection of LDH. Activity of the enzyme at room temperature (*curve 1*), and after incubation at 45 °C for 30 minutes: in buffer alone (*curve 2*), 2.5 mM spermidine (*curve 3*), 5 mM Spd (*curve 4*), 0.1 mg/ml PDC-109 (*curve 5*), 0.1 mg/ml PDC-109+2.5 mM Spd (*curve 6*) PDC-109+5 mM Spd are shown (*curve 7*). (**D**) Bar diagram representing the percent activity of LDH of panel E.

Table 1. Effect of spermine and PDC-109 on G6PD activity under thermal stress.

Additive	Percent activity
No additive	19
Spm 2.5 mM	31
Spm 5 mM	41
PDC-109 0.1 mg/ml	64
Spm 2. 5 mM + PDC-109 0.1 mg/ml	78
Spm 5 mM + PDC-109 0.1 mg/ml	89

Table 2. Effect of Spm/Spd and PDC-109 on LDH activity under thermal stress.

Additive	Percent activity
No additive	12
Spm 2.5 mM	27
Spd 2.5 mM	22
Spm 5 mM	38
Spd 5 mM	37
PDC-109 0.1 mg/ml	54
Spm 2.5 mM + PDC-109 0.1 mg/ml	73
Spd 2.5 mM + PDC-109 0.1 mg/ml	59
Spm 5 mM + PDC-109 0.1 mg/ml	93
Spd 5 mM + PDC-109 0.1 mg/ml	78

4.4 Polyamines Spm/Spd prevented thermal aggregation of LDH, aldolase and CA

Results of turbidimetric studies aimed at investigating the effect of polyamines on the ability of PDC-109 to inhibit the thermal aggregation of substrate proteins (like LDH, aldolase) are shown in Fig. 4. When LDH was incubated at 48 ^{o}C, the turbidity of the sample increased rapidly with time, reached a maximum and then levelled off (Fig. 4A, curve 1). Presence of Spm (2.5 mM and 5mM) alone reduced the extent of this aggregation to 85.29% and 67.65%, respectively, in a concentration-dependent manner (Fig.5A, curve 2 and 3). LDH to PDC-109 ratio

2:1 led to a considerable reduction in the rate of aggregation. The observed aggregation was 58.82% compared to that of the native enzyme in presence of PDC-109 alone. The aggregation decreased further when PDC-109 and Spm were used together. The aggregation was 47.05% and 29.42% in presence of 0.05 mg/ml PDC-109 with spermine concentrations 2.5 mM and 5mM, respectively, (Fig.4A, curve 5 and 6). Results of LDH aggregation in presence of different concentration of Spm alone and in combination with PDC-109 are summarized in Table 3.

Table 3. Effect of spermine and PDC-109 on thermal aggregation of LDH.

Additive	Percent aggregation
Spm 2.5 mM	85
Spm 5 mM	68
PDC-109 0.05 mg/ml	59
Spm 2.5 mM +PDC-109 0.05 mg/ml	47
Spm 5 mM + PDC-109 0.05 mg/ml	29

Table 4. Effect of Spm/Spd and PDC-109 on thermal aggregation of aldolase.

Additive	Percent aggregation
Spm 5 mM	73
Spd 5 mM	90
PDC-109 0.05 mg/ml	59
Spm 5 mM + PDC-109 0.05 mg/ml	24
Spd 5 mM + PDC-109 0.05 mg/ml	41

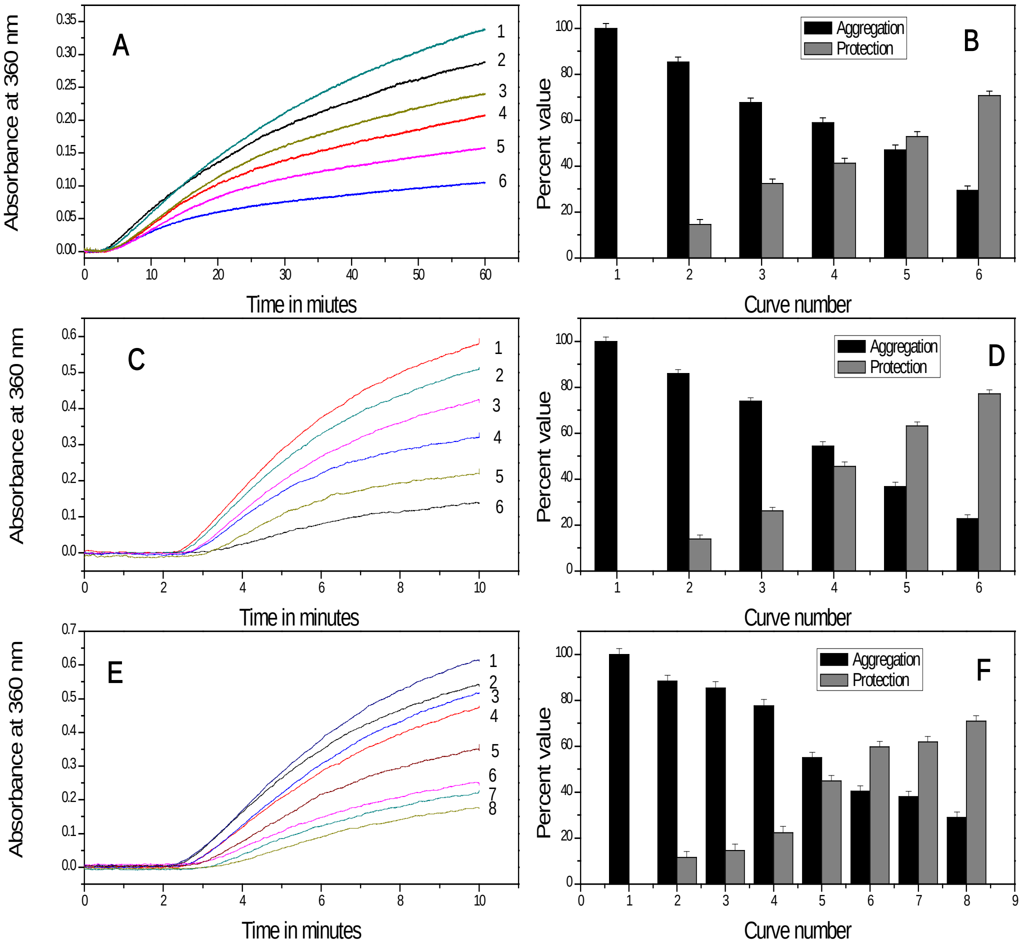


Fig.4. Chaperone-like activity of PDC 109. (A) Prevention of aggregation of LDH (0.1 mg/mL) by PDC-109 and spermine. Aggregation profiles of (*Curve 1*) LDH at room temperature, (*Curve 2*) LDH +2.5 mM spermine (*Curve 3*) LDH + 5 mM spermine (*Curve 4*) LDH+0.05 mg/mL PDC-109 and (*Curve 5*) LDH + 0.05 mg/mL PDC-109+2.5 mM spermine (*Curve 6*) LDH+0.05 mg/ml PDC-109+5 mM spermine are shown. **(B)** Bar diagram representing percent aggregation (black bars) and protection (gray bars) of LDH by PDC-109 and spermine at different concentrations. **(C)** Prevention of aggregation of Aldolase (0.1 mg/mL) by PDC-109 and spermine. Aggregation profiles of (*Curve 1*) Aldolase at room temperature, (*Curve 2*) Aldolase +2.5 mM spermine (*Curve 3*) Aldolase + 5 mM spermine (*Curve 4*) Aldolase+0.05 mg/mL PDC-109 and (*Curve 5*) Aldolase + 0.05 mg/mL PDC 109+2.5 mM spermine (*Curve 6*) Aldolase+0.05 mg/ml PDC-109+5 mM spermine are shown. **(D)** Bar diagram representing percent aggregation (black bars) and

protection (gray bars) of Aldolase by PDC-109 and spermine at different concentrations. **(E)** Prevention of aggregation of Aldolase (0.1 mg/mL) by PDC-109 and spermidine. Aggregation profiles of (*Curve 1*) Aldolase at room temperature, (*Curve 2*) Aldolase +5 mM spermidine (*Curve 3*) Aldolase + 7.5 mM spermidine (*Curve 4*) Aldolase+10 mM spermidine (*Curve 5*) Aldolase+0.05 mg/mL PDC-109 and (*Curve 6*) Aldolase + 0.05 mg/mL PDC-109+5 mM spermidine (*Curve 7*) Aldolase+0.05 mg/ml PDC-109+7.5 mM spermidine (*Curve 8*) Aldolase+0.05 mg/ml PDC-109+10 mM spermidine are shown. **(F)** Bar diagram representing percent aggregation (black bars) and protection (gray bars) of Aldolase by PDC-109 and spermidine at different concentrations.

To compare the effect of Spm and Spd on the CLA of PDC-109, turbidimetric assays on aldolase were performed independently with both the additives (Fig. 4C and E respectively). The result obtained when aldolase was used as a substrate protein were quite similar to those obtained with LDH. When incubated with 5 mM Spm and Spd aldolase aggregation levels were 73.1% (Fig. 4C, curve 3) and 90% (Fig. 4E, curve 2), respectively. Again, in the presence of the same concentration of both the additives (5mM) along with PDC-109 (0.05 mg/ml) the effect of Spm (aggregation 24%, Fig.4C, curve 6) was more compared to Spd (aggregation 40.65%, Fig. 4E, curve 6) in inhibiting the thermal aggregation of aldolase. Results for aggregation of aldolase in presence of Spm/Spd are summarized in Table 4.

The ability of spermine to prevent thermal aggregation of target proteins was further confirmed by AFM studies (Figure 5). The results obtained show that heat treated carbonic anhydrase forms large aggregates (Figure 5B). Presence of PDC-109 and Spm effectively prevented the aggregation of CA (Figure 5C and 5D). In the presence of either of them small particle like structures are present, which are similar to protein sample kept at room temperature (Figure 5A).

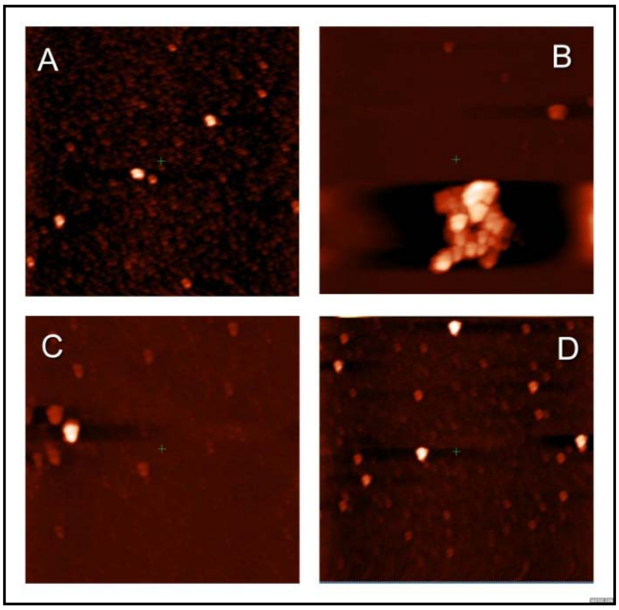


Fig. 5. AFM image of 40 µg/ml carbonic anhydrase (A) at room temperature (B) after incubation at 45 °C for 10 minutes (C) incubation at 45 °C for 10 minutes in presence of 20 µg PDC-109 (D) Incubation at 45 °C for 10 minutes in presence of 2.5 mM Spermine. All images are 5 × 5 µm in size.

5. Discussion

In the present study effect of polycationic molecules, spermine and its precursor spermidine was investigated on the functional activities of PDC-109, namely lipid efflux ability and CLA. Result obtained from erythrocyte lysis (Fig.1A and 1B) and confocal imaging of erythrocyte morphology (Fig. 1C, 1D, 1E and 1F) clearly show that the ability of PDC-109 for lipid efflux is increased in presence of Spm/Spd. These results were further confirmed by AFM imaging of DMPC model membrane and PDC-109 interaction. The AFM results show that in presence of Spm, the ability of PDC-109 to disrupt DMPC membrane increases significantly (Fig. 2). These results strongly suggest that in the presence of polyamines, the specificity of PDC-109 to choline phospholipids increases, with Spm being more effective than Spd. These results are of significant interest, as under *in vivo* condition lipid efflux is a crucial step during sperm capacitation. In an earlier EPR study it was shown that Spm binds to PC liposomes and it was suggested that the binding occurred by competition between positively charged ammonium group on spermine and positively charged ammonium group of choline for the negatively charged phosphate group (Momo *et al.*, 1995). PDC-109 binds to the choline group with high specificity and removes the lipid molecules from sperm plasma membrane. The possible explanation for the observed higher activity of PDC-109 in presence of spermine is that the binding of spermine to phosphate group in the backbone results in an increased binding affinity of the amino group of choline to interact with PDC-109 more strongly via cation–π interaction, resulting in higher membrane lysis ability of PDC-109.

To investigate the effect of Spm/Spd on the CLA of PDC-109, studies were carried out with many target proteins for their stability under thermal stress condition in presence of Spm/Spd. In a previous study it was shown that PDC-109 provides protection to these target proteins from such stress condition (Sankhala and Swamy, 2010). The present results show that G6PD and LDH are better protected against thermal stress when PDC-109 is used in combination with Spm/Spd than PDC-109 alone (Fig. 3). However, in the absence of PDC-109 these polycationic molecules themselves were able to provide protection to target proteins from thermal stress condition in concentration dependent manner.

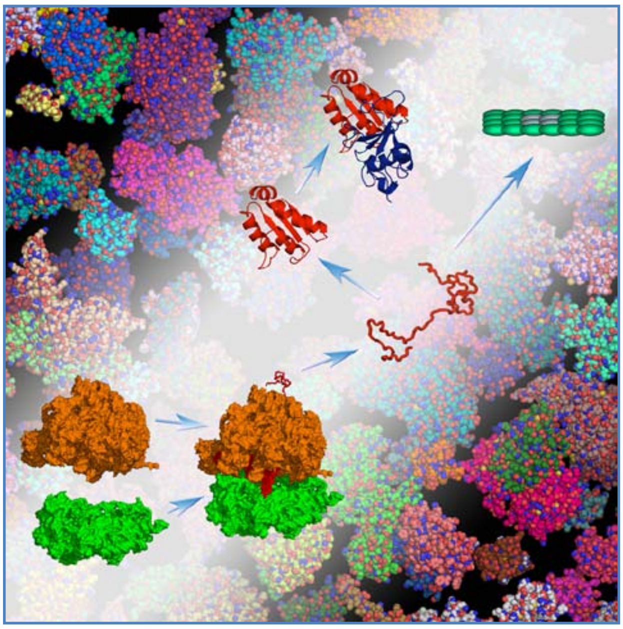
Many proteins are prone to self-aggregate at moderately high temperature. Under such condition they unfold from their native structure and self-aggregate with their exposed hydrophobic region (Chi *et al.*, 2003). In the present study we employed LDH, aldolase and CA to investigate the effect of these polycationic molecules on their self-aggregation. The results of these experiments show that when Spm/Spd are used in combination with PDC-109 self-aggregation of the above target proteins was less than in presence of PDC-109 alone (Fig. 4 and Fig. 5). Results from both the experiments, monitoring either enzyme activity or self-aggregation of target proteins show that Spm/Spd are able to protect target proteins from thermal stress condition and in presence of PDC-109 the protection observed is more than that expected for an additive effect, i.e., the two agents (polyamine and PDC-109) act in a synergistic manner. These results are significant as under *in vivo* condition proteins of seminal plasma come across harsh condition during the lengthy interval from insemination to fertilization (Yanagimachi**,** 1994). In germ-line specific cells presence of molecular chaperone such as calmegin and calnexin have been reported (Miller et al., 1992; Bergeron et al., 1994; Ikawa et al., 1997). However, there is considerable lack of knowledge about protecting mechanism for proteins of seminal plasma. Our results strongly suggest that polycationic molecules present at milimolar concentration in seminal plasma of many mammalian species work as chemical chaperones and protect the proteins of seminal plasma from denaturing surrounding environmental condition.

Many small molecules have been shown to prevent protein misfolding and aggregation under various denaturing conditions. Mechanism of their action is not well understood but many of these have been shown to be effective in treatment for various disease caused by protein misfolding and aggregation in model organism (Özcan et al., 2006; Cohen et al., 2003; Murray et al., 2014; Sawkar et al., 2002). In this regard, any small molecule which can prevent the proteins from misfolding and aggregation can be useful for potential therapeutic application. Knowledge of polycationic molecule for their ability to work as a chemical chaperone is not well established. Our study shows that at physiological concentration the polycationic molecules Spm and Spd effectively prevented the denaturation and aggregation of various proteins such as G6PD, LDH, aldolase and carbonic anhydrase under thermal stress, i. e., they work as chemical chaperones at physiological concentrations. Further, our results show these molecules are potential candidate to utilize for biotechnological application to prevent the protein aggregation during recombinant production and storage.

In summary, in the present work we investigated the effect of Spm and Spd on the lipid efflux ability and CLA of PDC-109. Our results show that presence of Spm/Spd enhances the lipid efflux ability of PDC-109 which facilitates sperm capacitation, and thus fertilization. Another important finding of the present study is that at physiological concentration these polycationic molecule work as a chemical chaperone which can provide protection to many proteins of reproductive system under *in vivo* denaturing condition for the successful fertilization.

Chapter 4

Molecular Crowding Differentially Affects the Chaperone-Like and Lipid Binding Activities of Major Protein of Bovine Seminal Plasma, PDC-109



Inside cells biochemical processes occur in crowded environments. The depicted processes include the binding of the large and small subunits of the ribosome, the folding of a nascent protein, its binding to another protein, and aggregation. The crowded conditions can significantly change the relative stability among the various species. Reproduced from *FEBS Letters* 587, 1053-1061.

1. Abstract

Effect of molecular crowding on the functional activities of the major bovine seminal plasma protein, PDC-109 was investigated. PDC-109 binds selectively to phosphatidylcholine (PC) on the sperm plasma membrane and also exhibits chaperone-like activity (CLA), thus enabling investigations on the effect of crowding on two distinctly different functions of the same protein. Presence of dextran (70 kDa) markedly increased erythrocyte lysis by PDC-109 in a concentration dependent manner. ITC studies showed that the affinity of PDC-109 to PC is higher in the presence of dextran. SEM studies showed that breakdown of PC membranes induced by PDC-109 was enhanced by the presence of dextran. These results strongly suggest that molecular crowding significantly enhances the binding of PDC-109 to choline phospholipids. To investigate the effect of molecular crowding on the CLA of PDC-109, the ability of PDC-109 to protect heat induced inactivation of glucose-6-phosphate dehydrogenase (G6PD) and heat induced aggregation of ADH and was monitored in the absence and presence of dextran at 48°C. The results obtained show that in crowding condition, the CLA of PDC-109 is reduced significantly. These result provide the first direct evidence that crowding can affect different function of same protein in different ways.

2. Introduction

The *in vivo* environmental conditions are significantly different from the *in vitro* experimental conditions which are used for study of biomolecules. The interior of cells, extracellular matrix and biological fluids contain a large variety of macromolecules, which are present at variable concentrations. Together these molecules occupy a significant fraction (10-40%) of the total aqua-based volume of the cell (Minton, 2003; Christiansen, 2013). Presence of such high concentration of biomolecules makes the *in vivo* environment crowded, which results in steric exclusion i.e. a fraction of the total space is physically inaccessible to other molecules. (Zimmerman and Minton, 1993; Minton, 2005; Zhou *et al.*, 2008). Numerous studies, theoretical and experimental, have demonstrated that chemical reactivity of biomacromolecules in crowded environments can be markedly different from that in dilute solutions which are typically used in laboratory experiments (Zimmerman and Minton, 1993; Ellis, 2001; Zhou *et al.* 2008, Batra *et al.*, 2009; Dhar *et al.*, 2010; Zhou, 2013). However, no study has been reported for effect of crowding condition on multifunctional protein i. e. how molecular crowding affects two or more function of a same protein.

Over the years a large number of theoretical and experimental studies have been carried out to investigate interactions of protein molecules in crowding condition, which has shed light on their behavior under such condition. However, most of these studies have focused on studying the effect of crowding on protein folding and stability (Zhou, 2004, Ai *et al.*, 2006; Zhou, 2008; Mukherjee *et al.*, 2009; Gershenson *et al.* 2010; Christiansen *et al.*, 2013). Similarly, a number of studies were also focused on investigating the effect of crowding on protein-protein interaction, for example, as in oligomer formation by many structural proteins, self-aggregation of proteins in different neurodegenerative diseases, in the formation of multiprotein complexes and so on (White *et al.*, 2010; Minton 2005b; Munishkina *et al*. 2004, Zorrilla *et al.*, 2004; Ellis, 2007, Uversky *et al.*, 2002; Hatters *et al.*, 2002, Alamo *et al.*, 2005; Gonzalez *et al.*, 2003, Huang *et al.*, 2010). Notwithstanding the immense importance of protein-ligand interactions in biological systems, studies aimed at investigating the effect of crowding on the binding of ligands to proteins have been rather scant.

As described in Chapter 1, PDC-109 is a multifunctional protein and binds to many small molecules including choline phospholipids, heparin and apolipoprotein A1 and A2 (Chandonnet *et al.* 1990; Desnoyers and Manjunath, 1992; *Manjunath et al.*, 2002). In the present work we used dextran (70 kDa) as a crowding agent to investigate the effect of molecular crowding on the lipid-binding ability of PDC-109. We also investigated the effect of molecular crowding on the CLA of PDC-109. The results obtained have clearly shown that molecular crowding differentially affects these two functions as was found to crowding increases the lipid-binding ability of PDC-109, but decrease its CLA.

3. Materials and Methods

3.1 Materials

Sephadex G-50 (superfine) was purchased from Pharmacia Biotech (Uppsala, Sweden). DEAE Sephadex A-25 was purchased from GE (Buckinghamshire, UK), DMPC was purchased from Avanti Polar Lipids. Phosphorylcholine (PrC), dextran (70 kDa), choline chloride, acrylamide, TEMED, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Sigma (St. Louis, MO, USA). Alcohol dehydrogenase, aldolase, tris base, EDTA, sodium chloride, and other chemicals were purchased from local suppliers and were of the highest purity available. PDC-109 was purified as described in Chapter 2. The purified protein was dialyzed extensively against 50 mM tris buffer, 0.15 M NaCl, 5 mM EDTA, pH 7.4 (TBS-I) and stored at 4°C.

3.2 Erythrocyte lysis assay

The effect of molecular crowding on the ability of PDC-109 to disrupt erythrocyte membranes was investigated as described in Chapter 2. From a stock solution of 400 mg/ml of dextran solution in tris buffer, appropriate amount was taken to make a 0, 50, 100 and 200 mg/ml of 1 ml sample, PDC-109 (100 µg) was added and the volume was adjusted to 800 µl with tris buffer. To this sample mixture, 200 µl of 4% erythrocyte suspension (in tris buffer) was added, properly mixed and kept for incubation for 1 h with intermittent gentle mixing. A control experiment was performed in a similar way with PDC-109 pre-incubated with 20 mM PrC. After 1 h of incubation, samples were centrifuged at 3000 rpm for 10 minutes, the supernatant was collected carefully and its absorption at 415 nm was recorded using a Cary 100 UV-Vis spectrophotometer (Agilent technologies, Santa Clara, CA, USA), which was also used for all other spectrophotometric measurments.

3.3 Preparation of DMPC model membrane and unilamellar vesicles

DMPC model membranes and unilamellar vesicles were prepared in the following manner. About 1.2 mg of the lipid was taken in a glass test tube and dissolved in about 1 ml of dichloromethane/methanol (1:1, v/v) mixture. The solvent was dried under a gentle stream of nitrogen gas followed by vacuum desiccation for at least 4 hours. The lipid film, thus formed on the glass surface, was hydrated with 1 ml of TBS-I buffer to obtain multilamellar vesicles (MLVS). To obtain unilamellar vesicles for ITC measurements, the MLVS were subjected bath sonication until a clear solution was obtained, indicating breaking down of the large multilamellar structures into unilamellar vesicles.

3.4 Scanning electron microscopy

To investigate the effect of molecular crowding on the interaction of PDC-109 with DMPC model membranes we performed field emission scanning electron microscopic studies using a MERLIN-Compact FESEM (ZEISS, Germany). From a stock solution of 1 mM of multilamellar vesicles of DMPC model membranes appropriate amount was taken to make the desired concentration of 150 µM and mixed with 0.5 mg/ml PDC-109 in TBS-I buffer containing 0, 50 and 100 mg/ml dextran and the final volume was adjusted to 1 ml. Samples were incubated for 20 minutes at room temperature. About 50 µl of sample was carefully deposited on freshly cleaved mica plates (1cm×1cm). To obtain a uniform thin film spin coating was done with 2000 rpm for 10 seconds, followed by 6000 rpm for 10 seconds. The thin film thus formed was washed with HPLC grade water and again spun at 2000 rpm for 10 seconds to remove water molecules and unbound membrane from the mica surface. The coated film was allowed to completely dry by keeping at room temperature for about 30 minutes, followed by placing it in a vacuum chamber for gold coating in Q150T ES gold coater (Quorum, East Sussex, UK) and then transferred to FESEM sample chamber for imaging.

3.5 Isothermal titration calorimetry

The interaction of PDC-109 with DMPC unilamellar vesicles was investigated in the presence and absence of crowding agent by isothermal titration calorimetry at 15 °C using a MicroCal VP-ITC instrument (MicroCal LLC, Northampton, MA, USA) as described previously (Anbazhagan *et al.*, 2011). Typically, 25 consecutive injections of 5 µl aliquots of the protein (250 µM) in presence of 100 mg/ml dextran were added with the help of a stirrer-syringe into the calorimeter cell of 1.445 ml filled with 80 µM of the DMPC unilamellar vesicles in presence of 100 mg/ml dextran. Samples were degassed under vacuum prior to their use in ITC experiments. To minimize the contribution of heat of dilution to the measured heat change, the protein solution and lipid vesicles were prepared in the same buffer. Injections were made at intervals of 5 minutes for all titrations. In order to ensure proper mixing after each injection, a constant stirring speed of 300 rpm was maintained during the experiment. To compare the binding affinity in the absence of crowding, experiments were also performed under similar conditions without dextran. Control experiments were performed by injecting PDC-109 solution into the buffer solution in an identical manner and the resulting heat changes were subtracted from the measured heats of binding.

3.6 Enzyme activity assay

Effect of molecular crowding on the CLA of PDC-109 was investigated using glucose-6-phosphate dehydrogenase (G6PD) as the target protein, by monitoring the activity of the enzyme activity by spectrophotometric method, as described in Chapter 3. In this assay G6PD converts G6P into 6-phospho-D-gluconate with simultaneous reduction of NADP to NADPH, which absorbs light at 340 nm. The reaction mixture contained 12 mM each of $MgCl_2$ and KCl, 5 mM G6P, 0.1 mM NADP and 0.5 µM G6PD. Reaction was initiated with the addition of NADP and increase in absorbance at 340 nm by NADPH was monitored. To investigate the effect of crowding on CLA of PDC-109 G6PD was incubated with PDC-109 (100 µg) at 45 °C for 45 minutes in the absence and presence of 100 mg/ml dextran. A higher concentration of PDC-109 (150 and 200 µg) was also used to monitor the enzyme activity under crowding condition. Relative activity of various samples was normalized with respect to the activity of G6P at room temperature.

3.7 Aggregation assay

To investigate the effect of molecular crowding on aggregation inhibition of target proteins by PDC-109, alcohol dehydrogenase (ADH) was used as a model system, and the experiment was carried out as described in Chapter 2. To study the effect of crowding on the ability of PDC-109 to protect the aggregation of ADH under thermal stress, a 0.1 mg/ ml sample of ADH was incubated with 0.05 and 0.1 mg/ml PDC-109 in the presence and absence of 100 mg/ml dextran for about 2-3 minutes. Samples were transferred to a 1 ml cuvette and placed in the sample holder of the spectrophotometer which was maintained at 48 °C. After a 30 second equilibration period, absorption at 360 nm was monitored for 10 minutes. In a control experiment aggregation of 0.1 mg/ml ADH was monitored in the absence of PDC-109. Percent aggregation of each experimental sample was normalized with respect to aggregation of ADH in absence of PDC-109 (Control).

3.8 Fluorescence spectroscopy

The effect of molecular crowding on the surface hydrophobicity of PDC-109 was investigated by fluorescence spectroscopy, by monitoring binding of the hydrophobic probe bis-ANS to the protein. Fluorescence spectra were recorded on an ISS PC1 fluorescence spectrometer (Champaign, IL, USA). A 10 µM solution of PDC-109 was mixed with 10 µM of bis-ANS in the presence of 0, 100 and 200 mg/ml dextran and incubated for about 2-3 minutes. The sample was excited at 390 nm and spectra were recorded from 400-600 nm keeping the excitation and emission bandpass at 3 and 5 nm, respectively. In a control experiment bis-ANS was added to the sample buffer and spectra were recorded in absence of the protein.

3.9 Differential scanning calorimetry

The effect of crowding on the thermal stability of PDC-109 was investigated by DSC as described in Chapter 2 for rPDC-109. Experiments were carried out on a MicroCal VP-DSC apparatus (MicroCal LLC, Northampton, MA, USA). PDC-109 with a 0.8 mg/ml concentration was subjected to thermal scan between 10 and 90 °C at a scan rate of 40 °C/h in tris buffer in the presence of 100 mg/ml dextran. Before scanning, both sample and reference solutions were properly degassed to ensure that the samples are free from any air bubbles. Baseline was obtained with tris buffer in the presence of 100 mg/ml dextran and reproducibility was verified with multiple scans. Buffer scans were subtracted from thermograms of PDC-109. The DSC data were analysed using Origin 7.0 software provided with the instrument using non-two-state transition model.

4. Results

4.1 Molecular crowding increases erythrocyte lysis ability of PDC-109

Incubation of erythrocyte mixture with PDC-109 results in lipid efflux from its plasma membrane, which results in membrane disruption and release of haemoglobin into the solution. The absorbance at 415 nm (due to released haemoglobin) can be correlated to the extent of membrane disruption. Effect of crowding on erythrocyte lysis is shown in Figure 1. From this figure it is clear that ability of PDC-109 to disrupt the plasma membrane increases with increase in the dextran concentration. Control experiments performed with PDC-109 pre-incubated with 20 mM PrC showed negligible erythrocyte lysis, indicating that dextran alone does not induce erythrocyte lysis.

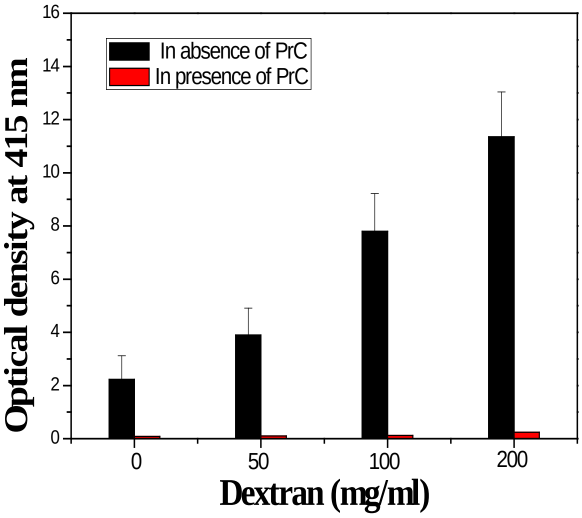


Figure 1. Erythrocyte lysis assay. A 0.8% human erythrocyte solution was incubated for 1 h with 100 µg of PDC-109 in presence of 0, 50, 100 and 200 mg/ml dextran. In control experiment PDC-109 was preincubated with 20 mM PrC.

4.2 Molecular crowding increases the disruption of DMPC model membrane by PDC-109

The above observation that crowding increases the lipid efflux ability of PDC-109 was further confirmed by SEM imaging of PDC-109/DMPC model membrane interaction (**Fig. 2**). In buffer alone DMPC shows multilamellar structures (**Fig. 2A**). Incubation for 20 minutes with 0.5 mg/ml PDC-109 shows disruption of the well defined multilamellar structure of DMPC, with breakage of lamellar structure at several places (**Fig. 2B**). Samples incubated with 50 mg/ml dextran show the loss of multilamellar structure, with the membrane being broken into smaller patches (**Fig. 2C**). Increasing the dextran concentration to 100 mg/ml results in further fragmentation of the small patches of membrane into even smaller pieces

(**Fig. 2D**). These results show that moderate level of crowding significantly increases the lipid binding and membrane disruption ability of PDC-109.

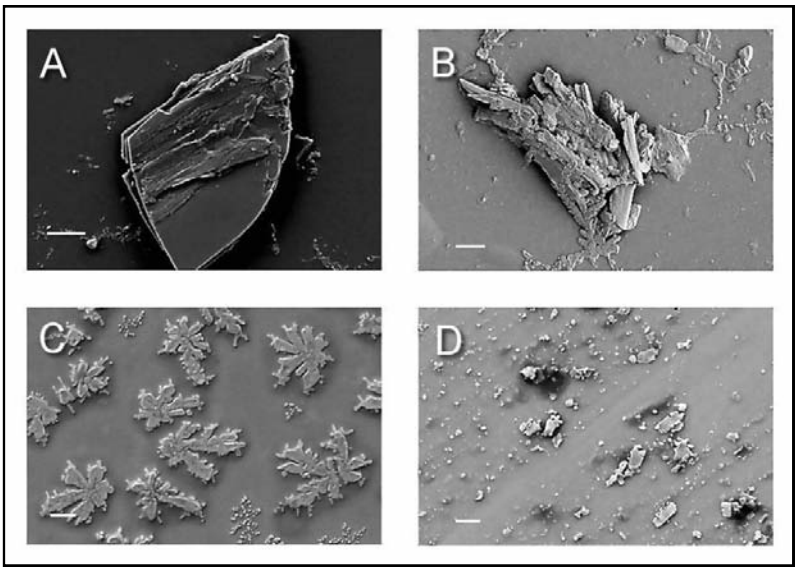


Figure 2: SEM imaging of interaction of DMPC model membrane and PDC-109. Images showing 150 µM DMPC model membrane incubated at room temperature for 20 minutes in (A) Buffer alone, (B) 100 µg PDC-109 in buffer, (C) 100 µg PDC-109 and 50 mg/ml dextran in buffer, and (D) 100 µg PDC-109 and 100 mg/ml in buffer. Scale bar 2 µM.

4.3 Molecular crowding increases binding affinity of PDC-109 with DMPC vesicles

The above results clearly shows that under crowding condition the lipid-efflux ability of PDC-109 is significantly higher. To investigate the effect of crowding on choline phospholipid binding to PDC-109 we performed ITC experiments with DMPC unilamellar vesicles. These experiments show that in buffer alone PDC-109 interacts with DMPC model membrane with a K_b of 2.94×10^6 M^{-1}(**Fig 3A**), whereas in the presence of 100 mg/ml dextran, the binding strength increases and the K_b obtained is 9.4×10^6 (**Fig. 3B**). Thus, the ITC studies show that under crowding condition affinity of PDC-109 for choline phospholipids is significantly higher and suggest that the observed higher lipid-efflux ability of PDC-109 under crowding condition most likely results from the higher affinity of the protein for choline phospholipids.

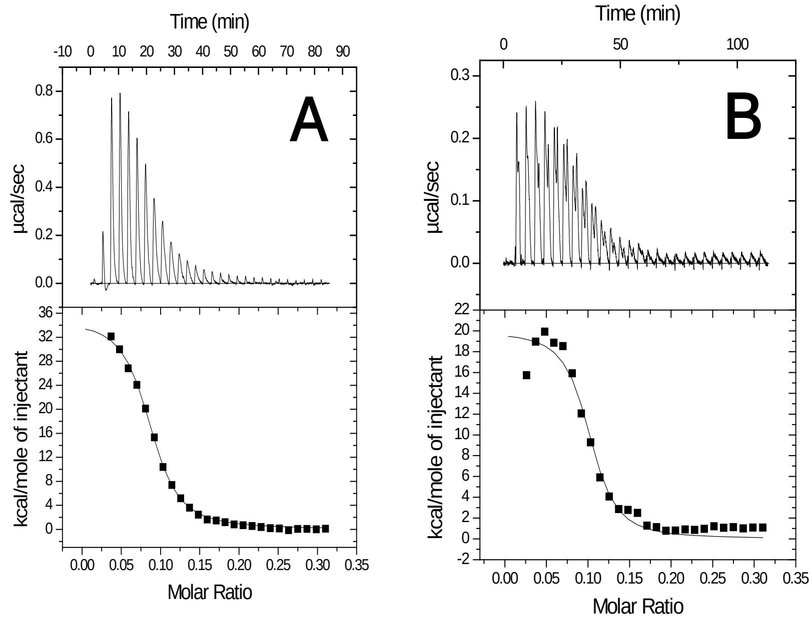


Figure 3: ITC study of PDC-109 binding with DMPC model membrane (A) In buffer **(B)** In presence of 100 mg/ml dextran. Upper panel show raw titration data and lower panels show integrated heats of binding obtained from the data shown in the upper panels. See text for details.

4.4 Crowding condition stabilize oligomeric form of PDC-109

PDC-109 is an oligomeric protein. As theoretical models and experimental studies show that molecular crowding increases oligomerization and self-assembly of proteins (Zhou, 2013; Snoussi and Halle, 2005; Aguilar *et al.*, 2011), we carried out DSC studies on PDC-109 in presence of 100 mg/ml dextran (Fig. 4).

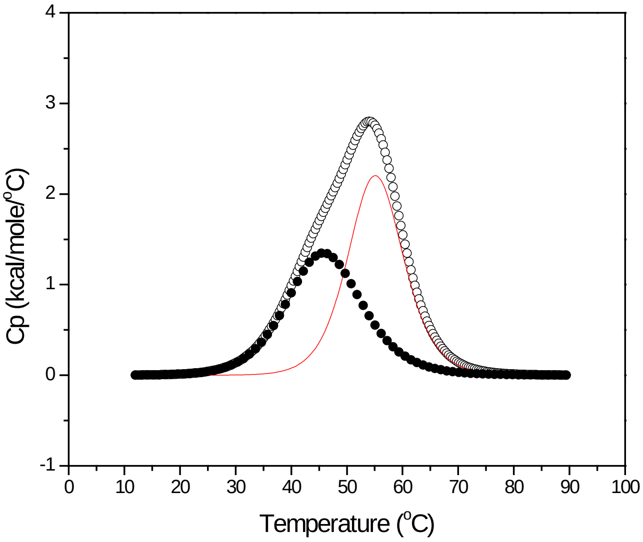


Figure 4: DSC study of PDC-109 in presence of 100 mg/ml dextran. Open circle showing original scan. Closed circle showing first transition temperature for monomerization of PDC-109 oligomers and solid line showing second transition corresponding to complete unfolding of monomeric species of PDC-109.

Previous work has shown that the DSC thermogram of PDC-109 is characterized by two transitions. The first transition occurs at about 309-310 K and corresponds to dissociation of the oligomeric species to yield monomers, whereas the second transition occurs at about 328 K and corresponds to unfolding of the monomer (Gasset *et al.*, 1997; Sankhal *et al.*, 2011). The present result obtained under crowding condition show that unfolding temperature of the monomers is ~328 K, which matches well with previous reports. However, the temperature corresponding to the dissociation of the oligomeric form is markedly increased from 310 K to 319 K (**Fig. 4**). This demonstrates that crowding condition stabilizes the oligomeric form of the protein.

4.5 Under crowding condition ability of PDC-109 to protect heat induced denaturation of G6PD decreases

When incubated in Tris buffer at 45 °C for about 45 minutes G6PD shows only about 30 percent activity as compared to that observed at room temperature (Fig. 5A). Incubation of G6PD with 6-7 µM PDC-109 for the same time period at 45 °C shows more than 80 percent activity. When G6PD was incubated at 45 °C in the presence of 100 mg/ml dextran, again its activity decreased to about 30 percent of its residual activity (Fig. 5B). This shows under buffer condition and in crowding condition temperature has same effect on G6PD activity. However incubation of G6PD at 45 °C for 45 min in the presence of 6-7 µM PDC-109 and 100 mg/ml dextran resulted in only marginal increase in the activity as compared to that without PDC-109. When PDC-109 concentration was increased to 10-11 µM G6PD activity increased to 40-50 percent As compared to that seen at room temperature, whereas further increase of PDC-109 concentration to 15 µM resulted in an increase in the activity to about 60 percent of its activity. These results clearly indicate that under crowding condition the ability of PDC-109 to protect the G6PD from thermal stress condition is significantly reduced.

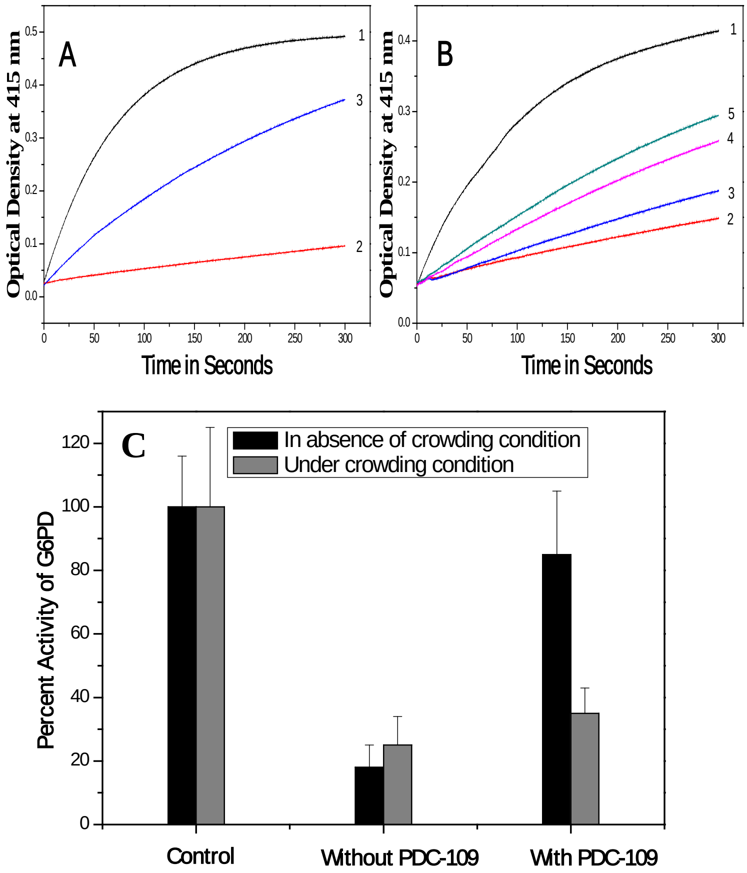


Figure 5: Effect of crowding on CLA of PDC-109 against thermal denaturation of G6PD. (A) G6PD activity in buffer, *curve 1* at RT, *curve 2* upon incubation of G6PD at 45 °C for 45 minutes and *curve 3* upon incubation of G6PD with PDC-109 at 45 °C for 45 minutes. **(B)** G6PD activity in presence of 100 mg/ml dextran in buffer, *curve 1* at RT, *curve 2* after incubation at 45 °C for 45 minutes, *curve 3*, *4* and *5* after incubation at 45 °C for 45 minutes in presence of 0.1, 0.15 and 0.2 mg/ml PDC-109 respectively. **(C)** Bar diagram representing percent activity of G6PD after incubation at 45 °C for 45 minutes in the absence presence of 0.1 mg/ml PDC-109. Black bar shows percent activity in buffer alone and gray bar shows activity in presence of 100 mg/ml dextran. Column at extreme left shows activity of G6PD at room temperature, which has been taken as 100 % to calculate relative activity of other samples.

4.6 Under crowding condition ability of PDC-109 to prevent heat induced aggregation of ADH decreases

Aggregation profile of heat treated alcohol dehydrogenase was monitored by spectrophotometric method as described above. When ADH at a final concentration of 0.1 mg/ml was used, optical density of the sample reaches a mamimum of about 0.4 in about 300 seconds and then levels off (Fig. 6A, *curve 1*). When ADH at the same concentration was incubated with 0.05 mg/ml of PDC-109 aggregation was only about 30 % (Fig. 6A, *curve 2*).

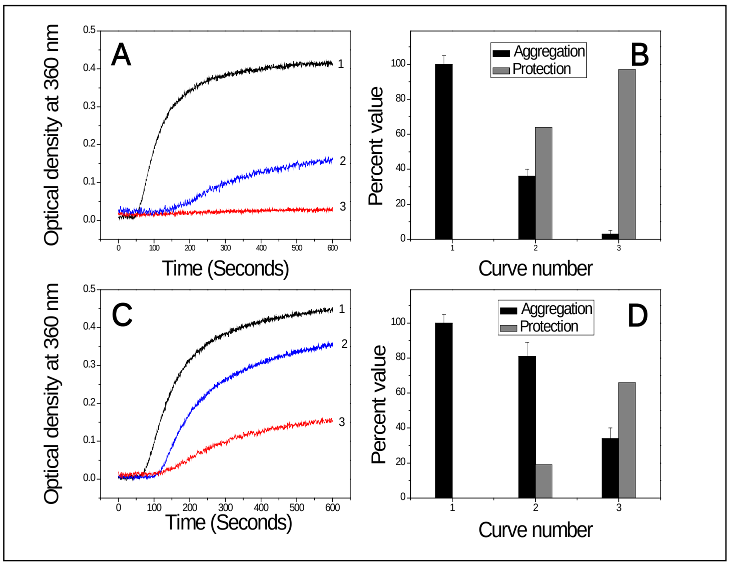


Figure 6. Effect of molecular crowding on CLA of PDC-109 to protect heat induced aggregation of ADH (A) ADH aggregation assay in buffer, *Curve 1* aggregation of 0.1 mg/ml ADH at 48 °C, *curves 2* and *3,* thermal aggregation of 0.1 mg/ml ADH in presence of 0.05 and 0.1 mg/ml PDC-109. **(B)** Bar diagram representation for percent aggregation and percent protection of data shown in fig. 6A. **(C)** ADH aggregation assay in buffer and in presence of 100 mg/ml dextran, *curve 1* aggregation of 0.1 mg/ml ADH in buffer and in presence of 100 mg/ml dextran, *curves 2* and *3* aggregation of 0.1 mg/ml ADH in presence of 0.05 and 0.1 mg/ml PDC-109 in buffer and in presence of 100 mg/ml dextran. .**(D)** Bar

diagram representation for percent aggregation and percent protection of data shown in fig. 6C.

Increasing the concentration of PDC-109 to 0.1 mg/ml resulted in the near complete inhibition of ADH aggregation (Fig. 6A, *curve3*). When these experiments were performed in presence of 100 mg/ml dextran there was a slight increase in the absorbance as compared to that in the absence of crowding agent (in absence of PDC-109) (Fig. 6C, *curve 1*). Incubation of ADH with 0.05 mg/ml PDC-109 shows that at this concentration aggregation was about 80% (Fig. 6C, *curve 2*). Increasing the concentration of PDC-109 to 0.1 mg/ml resulted in an aggregation of about 35% (Fig. 6C, *curve 3*). These results show that molecular crowding significantly reduces the ability of PDC-109 to prevent thermal aggregation of ADH. Bar diagrams representing the percent aggregation and percent protection under different conditions are shown in Figures 6B and 6D in absence and presence of crowding condition, respectively.

4.7 Molecular crowding decreases the surface hydrophobicity of PDC-109

Surface hydrophobicity plays an important role in the chaperone like activity of proteins. To understand the effect of crowding on the observed decrease in the CLA of PDC-109, its surface hydrophobicity was investigated using the hydrophobic probe bis-ANS (Fig. 7). The results obtained indicate that in the presence of 100 mg/ml dextran, the surface hydrophobicity of PDC-109 is about 80% (Fig. 7, *curve 3*) as compared to that in buffer alone (Fig. 7, *curve 2*). Increasing the dextran concentration to 200 mg/ml resulted in a further decrease in the hydrophobicity to about 62% of that observed with the protein in buffer alone (Fig. 3, *curve 4*). It can be inferred from these results that under crowding condition surface hydrophobicity of PDC-109 decreases, which in turn reduces its CLA.

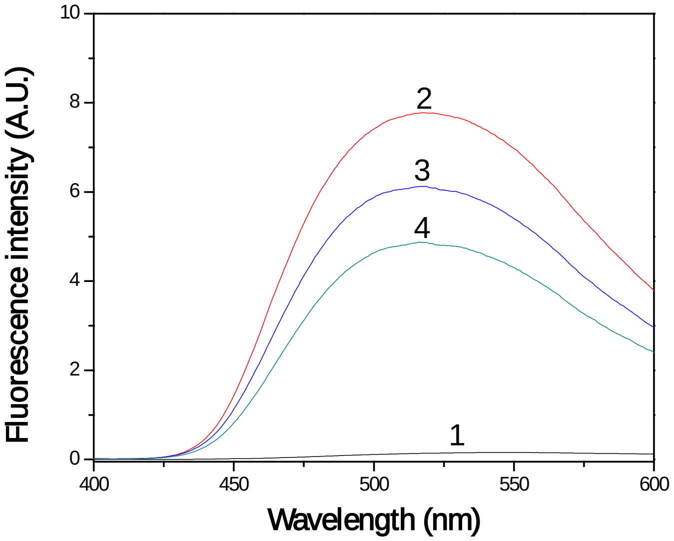


Figure 7. Effect of crowding on surface hydrophobicity of PDC-109. Fluorescence spectra of bis-ANS in buffer (*curve1*), in presence of 10 µM PDC-109 in buffer (*curve 2*) and in presence of 10 µM PDC-109 in buffer with 100 and 200 mg/ml dextran (*curves 3* and *4*, respectively). Excitation wavelength was 390 nm and excitation and emission band passes was 2 and 5 nm, respectively.

Discussion

Molecular crowding affects physicochemical properties of biomolecules primarily through excluded volume effect (Ellis, 2001, Gnutt, 2015) and nonspecific soft interaction between macromolecule **(**Gennes, 1979; Minton, 2005, Groen *et al.*, 2015**)**. Excluded volume favours folded form of protein which in turn favours protein stability, whereas non-specific interaction with background macromolecules can be stabilizing, unaffecting or destabilizing for proteins (Hong and Gierasch, 2010; Miklos *et al.*, 2010; Miklos *et al.*, 2011). Under *in vivo* conditions both these factors operate simultaneously. To understand the effect of crowding on protein stability more thoroughly, an important study has been done using poly(vinylpyrrolidone) (PVP) as a crowding agent with emphasis on residual effect on protein molecule i. e., effect of individual residues in protein molecules and their spatial location on the protein stability. Results of the study showed that protein stability depends on both spatial location of individual residues in the protein and concentration of the crowding agent (Charlton *et al.*, 2008). Thus, for the stability of a protein in crowding condition various factors play important roles and stability of the protein is the outcome of all these factors and it can vary from protein to protein, which in turn determines the behaviour of the protein molecules.

To understand the behaviour of PDC-109 under crowding condition we used dextran (70 kDa) as a crowding agent. Results obtained from erythrocyte lysis assay (**Fig. 1**) and interaction of DMPC model membrane with PDC-109 (**Fig. 2**) clearly showed that under crowding condition, the ability of PDC-109 to induce lipid efflux increases significantly. To understand whether the observed increase in the activity of PDC-109 is due to effective higher concentration of reactants or due to increased binding affinity of reactants, we carried out ITC studies on the interaction of PDC-109 with DMPC vesicles. The results obtained show that under crowding condition the affinity of DMPC-PDC-109 interaction is significantly higher. Therefore, the observed higher ability of PDC-109 to induce lipid-efflux appears to be due to increase in its affinity for lipid molecules. These results are significant as under *in vivo* condition lipid-efflux from sperm plasma membrane at the time of acrosome reaction is a crucial event before fertilization.

Many proteins are known to act as extracellular chaperones besides their specific biological function. Examples include bovine serum albumin, clusterin, haptoglobin, and αS-casein (Finn *et al.* 2012; Humphreys, 1999; Wyatt *et al.*, 2011; Sultan *et al.*, 2013, Wyatt *et al.*, 2013). These proteins plays protective roles and help maintain the proper functioning of many extracellular proteins. Extracellular matrix and biological fluids contain high concentrations of proteins and various metabolites, which occupy between 10% to 40% of the total volume (Minton, 2003; Christiansen, 2013). Therefore it is important to know the effect of crowding condition on CLA of theses extracellular chaperones. We utilized thermal inactivation of G6PD and heat induced aggregation of ADH to evaluate the effect of crowding on the CLA of PDC-109. The results obtained indicate that molecular crowding decreases the CLA of PDC-109 significantly. In the last few decades a number of studies have been carried out to understand the mechanism of CLA of proteins, Despite this, the mechanism of CLA at molecular level is poorly understood. In this regard, it is important to note that several important studies shows that at higher temperature higher oligomeric forms of protein dissociate into smaller units, which is important for their enhanced CLA (Montfort et al., 2001; Haslbeck et al., 1999; Gu et al., 2002; Haslbeck et al., 2008; Jaya et al., 2009; Shashidharamurthy et al., 2005; Franzmann et al., 2008; Benesch et al., 2008). Dissociation of higher oligomeric form at elevated temperature results into exposure of their buried hydrophobic region by which they interact with hydrophobic region of partially denatured client proteins and protect them from denaturation (Lee *et al.*, 1997; Sharma *et al.*, 1998; Haslbeck 2005). The results of our DSC studiesshow in presence of crowding agent the dissociation of oligomeric form of PDC-109 markedly increases from 37 °C to 46°C, that is, crowding stabilizes the oligomeric form of PDC-109 at higher temperature. Thus according to the above model which suggest that dissociation of higher oligomeric form is important for CLA at higher temperature, PDC-109 should show reduced CLA at higher temperature, which is consistent with our experimental results from G6PD activity assay and ADH aggregation assay. Results obtained from bis-ANS binding studies show that crowding decreases the surface hydrophobicity of PDC-109. It is known for many well established small heat shock proteins that surface hydrophobicity plays an important role for CLA (Raman and Rao, 1994; Mendoza et al., 1991; Das et al., 1995). The observed decrease in CLA of PDC-109 under crowding condition most probably results from the presence of undissociated form of protein and decrease in surface hydrophobicity of PDC-109.

In summary, the present study was carried out to investigate the effect of molecular crowding on two markedly different functions of PDC-109 using dextran-70 as a crowding agent. To the best of our knowledge this is first study to investigate the effect of crowding on two different functions of the same protein. Result obtained from the present study show that molecular crowding significantly increases the lipid-binding ability of PDC-109 and drastically reduces the CLA. Thus, the present results shows that molecular crowding can have differential effect on two different function of the same protein. In this regard, studies on other multifunctional proteins under crowding condition will be helpful to understand many fundamental biological processes in the physiological milieu.

Chapter 5

General Discussion and Conclusions

The work embodied in this dissertation entitled **"Mutational analysis of PDC-109 and effect of polyamines and molecular crowding on its functional activities"** covers several new aspects of study of PDC-109. Among mammalian seminal plasma proteins, PDC-109 is one of the most extensively studied and well characterized (Esch *et al.*, 1983; Manjunath and Sairam, 1987; Desnoyers and Manjunath, 1992; Damai *et al.*, 2010; Gwathmey *et al.*, 2003; Gasset *et al.*, 1997; Swamy, 2004; Sankhala and Swamy, 2010), which provided rich source of background information to carry out the present work in new dimensions.

Content in Chapter 2 contains the work carried out to understand the role of N-glycosylation of PDC-109 and importance of a highly conserved core Trp residue across all FnII domains present in a variety of proteins. For this purpose PDC-109 was cloned in pet 21a vector and expressed in *E. Coli* expression system. Different mutants (W47A, W93A and W106A) were made by PCR based site-directed mutagenesis and their sequences were confirmed by gene sequencing. As all the cloned protein came into inclusion bodies, protein folding was done using GSH/GSSH redox system. Proper folding of rPDC-109 and mutants was confirmed by CD spectral analysis, which showed that these proteins were folded properly with minimal structural changes.

Studies were carried out to investigate the role of N-glycosylation using various approaches. DSC thermogram of rPDC-109 shows that glycosylation of PDC-109 provides a moderate stability to it from thermal denaturation. A comparison of fluorescence spectra of rPDC-109 with WT- PDC-109 in the absence and presence of various ligands shows that in the presence of ligand percent enhancement of fluorescence maximum is slightly higher than that observed with WT PDC-109. Erythrocyte lysis assay and confocal imaging of erythrocyte morphology in the presence of WT- and rPDC-109 show that rPDC-

109 is more efficient than WT PDC-109 in causing lipid-efflux from plasma membrane of erythrocytes, whereas analysis of ADH aggregation assay in the presence of WT- and rPDC-109 show that the CLA of rPDC-109 is less than that of WT PDC-109. From these studies it was concluded that glycosylation modulates the functional activities of PDC-109 by decreasing its lipid-efflux ability and increasing the CLA.

The significance of conserved core Trp residue in the FnII domains of PDC-109 was revealed by mutational analysis. Erythrocyte lysis assay and confocal imaging of erythrocytes in the presence of different mutants show that while mutant W47A exhibits significantly reduced lipid-efflux ability (~ 10% as compared to rPDC-109), mutations in the second FnII domain of PDC-109 resulted in a complete loss of lipid-efflux ability. These results show that the conserved Trp residue in FnII domains of PDC-109 are important for its lipid-efflux ability. To investigate the effect of these mutations on the CLA of PDC-109, ADH aggregation was used as a model system, which showed that only mutant W47A was able to prevent the ADH aggregation from thermal denaturation but with a significantly reduced ability as compared to rPDC-109. Mutant W93A and W106A did not show any CLA. On the contrary in presence of mutants W93A and W106A the observed increase in optical density was more than that observed with ADH alone. To understand the observed higher optical density in presence of mutants W93A and W106A, these mutants were tested by turbidimetric assay for self-aggregation. These experiments reveled that in absence of target protein these proteins do not self aggregate. Thus it appears that in presence of the target protein the observed higher optical density is due to co-aggregation of these mutants along with the target protein. It is known that surface hydrophobicity and oligomeric status are two important factors for the CLA of proteins (Raman and Rao, 1994; Mendoza *et* *al.*, 1991; Das *et al.*, 1995; Chang *et al.*, 1996; Van *et al.*, 2001). Thus these two properties of mutant proteins were investigated to understand the observed loss of CLA. Fluorescence spectroscopic experiments with the hydrophobic probe bis-ANS showed that all the mutants acquired higher surface hydrophobicity as compared to rPDC-109, which suggest that they should exhibit better CLA. However, cross-linking of proteins with glutaraldehyde showed that mutation of these residues resulted in the loss of oligomeric status of the protein. As oligomeric status is important for CLA of small heat shock proteins, the observed loss of CLA could be due to the loss of oligomeric nature of the protein. These results are significant, as FnII domains are structural component of many proteins, some of which play important roles in a number of fundamental biological processes. Therefore, the knowledge obtained from the present study is expected to be useful to understand the structural basis of functional role played by other proteins with FnII domains.

The result presented in Chapter 3 deal with studies on the functional activities of PDC-109 in the presence of Spermine (Spm) and Spermidine (Spd). Spm is a component of seminal plasma of many mammalian species and had been shown to bind to sperm plasma membrane (Atmar *et al.*, 1981; Rubinstein *et al.*, 1991; Rubinstein *et al.*, 1995). PDC-109 is well characterized with respect to its interaction with lipids of the sperm plasma membrane and its lipid-efflux ability from the plasma membrane containing choline phospholipids (Swamy, 2004). As both Spm and PDC-109 interact with sperm plasma membrane, in the present study we carried out experiments to investigate the effect of Spm and its precursor Spd on the functional activities of PDC-109. The result obtained from these studies show that in presence of Spm/Spd the lipid-efflux ability of PDC-109 increases in a concentration dependent manner. Thus, the presence of high concentration of these polycationic molecules in the seminal plasma appears to play an important role in sperm capacitation by increasing the lipid-efflux ability of PDC-109. To investigate the effect of Spm/Spd on the CLA of PDC-109 several target proteins such as G6PD, LDH, carbonic anhydrase and aldolase were used as target proteins and their thermal denaturation profiles in presence of different combinations of Spm/Spd with PDC-109 were characterized. The results obtained show that in presence of Spm/Spd the CLA of PDC-109 is higher. Use of Spm/Spd alone in the absence of PDC-109 shows these small polycationic molecules themselves are capable of protecting the target proteins from thermal denaturation; that is, they work as chemical chaperones. These result are significant, as small molecules working as a chemical chaperone can be potentially useful for therapeutics and in biotechnological applications.

Another important part of the present study, presented in Chapter 4 is the investigation of the effect of molecular crowding on the functional behavior of PDC-109. Numerous studies, theoretical and experimental, have demonstrated that chemical reactivity of biomacromolecules in crowded environments can be markedly different from that in dilute solutions used typically in laboratory experiments (Zimmerman and Minton, 1993; Ellis, 2001; Zhou *et al.* 2008, Batra *et al.*, 2009; Dhar *et al.*, 2010; Zhou, 2013). The present study was carried out to investigate, how crowding affects the different functions of the same protein. For this purpose we investigated effect of crowding on CLA and lipid-efflux ability of PDC-109. We used dextran (70 kDa) as a crowding agent for all experiments. Results from erythrocyte lysis assay and SEM study with model lipid membranes clearly demonstrated that under crowding condition lipid-efflux ability of PDC-109 increases significantly. Further experiments were done using ITC to investigate the interaction of DMPC unilamellar vesicles with PDC-109. These experiments showed that under crowding condition the affinity of PDC-109 for choline phospholipids increases significantly and suggest that the observed higher lipid-efflux ability of PDC-109 is due to an increase in the affinity of PDC-109 for the lipid molecules. To investigate the effect of crowding on CLA of PDC-109 we utilized G6PD activity assay and ADH aggregation assay. Results from these experiments show that under crowding condition CLA of PDC-109 significantly reduces. As surface hydrophobicity has been shown to play an important role for the CLA of small heat shock proteins, we investigated the surface hydrophobicity of PDC-109 using the hydrophobic probe bis-ANS. Results of these experiments show that increase in molecular crowding decreases the surface hydrophobicity of PDC-109. This suggests that the observed decrease in the CLA of PDC-109 under crowding condition is due to a decrease in the surface hydrophobicity of PDC-109. Thus, this study shows that molecular crowding increases the lipid-efflux ability of PDC-109 which is significant under *in vivo* conditions as lipid-efflux is an important step prior to fertilization. Another important feature of this study is that it shows crowding condition can affect two different functions of same protein in different ways.

Perspective of future direction of present study

The present study provides several significant findings which will be immensely useful to carry out further investigation to utilize them for various application purposes. Results presented in Chapter 2 show that the highly conserved core Trp residues in the FnII domains are important for ligand-binding by PDC-109. As these Trp residues are highly conserved across all FnII domains, similar mutational analysis of the FnII domains in other proteins can shed light on the importance of conserved core Trp residues in them. Such studies can lead to potential applications in therapeutics as many proteins containing FnII domains play important role in some of the fundamental processes in biological systems. One interesting example is binding of the bacterium *Campylobacter jejuni* to fibronectin in extracellular matrix. This strain of bacteria is responsible for one of the most common causes of human gastroenteritis in the world. It uses its FnII domain of transmembrane protein CadF to bind fibronectin (Larson *et al.*, 2013). Thus a knowledge of the structural basis of FnII domain function is expected to be promising for development of drugs which can be useful in treating gastroenteritis.

Results reported in Chapter 3 show that the polycationic molecule spermine and its precursor spermidine enhance the lipid efflux ability of PDC-109, which is important for fertilization.Our studies also show that at physiological concentration these molecules work as chemical chaperones. This is very significant finding as small molecules which possess the ability to prevent protein misfolding and prevent self-aggregation of denatured proteins have high potential for use in a number of biotechnological applications. Many small molecules with chaperone activity have been shown to be useful in animal models for curing many diseases arising from protein misfolding. Presently a few small molecules, which act as a chemical chaperones e. g. 4-phenylbutyric acid (PBA) and tauroursodeoxycholic acid (TUDCA) have been approved by US FDA and are being used for treatment of some of the human diseases (Engin and Hotamisligil, 2010). Also, because of their small size they can easily cross the blood brain barrier. Thus, they are potential candidates for treating neurological disorders arising from protein misfolding. In this regard Spm and Spd can be explored for their application for treating such diseases as they are small polycationic molecule and our results show that they behave as chemical chaperone.

Investigations carried out on PDC-109 function under crowding condition (reported in Chpater 4) shed light on how different functions of the same protein are affected by molecular crowding. These studies show that crowding can affect different function of same protein in different ways. Future investigation with other multifunctional protein will be helpful to understand many fundamental processes in the physiological milieu.

References

Aguilar, X., Weise, C. F., Sparrman, T., Wolf-Watz, M. and Wittung-Stafshede, P. (2011) Macromolecular crowding extended to a heptameric system: the cochaperonin protein 10. *Biochemistry* 50, 3034–3044.

Ahmed, A. K., Schaffer, S. W. And Wetlaufer, D. B. (1975) Nonenzymic reactivation of reduced bovine pancreatic ribonuclease by air oxidation and by glutathione oxidoreduction buffers. *J. Biol. Chem.* 250, 8477-8482.

Ai, X., Zhou, Z., Bai, Y. and Choy, W. Y. (2006) 15N NMR spin relaxation dispersion study of the molecular crowding effects on protein folding under native conditions. *J. Am. Chem. Soc.* 128, 3916–3917.

Alamo, M. D., Rivas, G. and Mateu, M. G. (2005) Effect of macromolecular crowding agents on human immunodeficiency virus type 1 capsid protein assembly in vitro. *J. Virol.* 79, 14271–14281.

Anbazhagan, V. and Swamy, M. J. (2005) Thermodynamics of phosphorylcholine and lysophosphatidylcholine binding to the major protein of bovine seminal plasma, PDC-109. *FEBS Lett.* 579, 2933–2938.

Anbazhagan, V., Damai, R. S., Paul, A. and Swamy, M. J. (2008) Interaction of the major protein from bovine seminal plasma, PDC-109 with phospholipid membranes and soluble ligands investigated by fluorescence approaches. *Biochim. Biophys. Acta* 1784, 891-899.

Anbazhagan, V., Sankhala, R. S., Singh, B. P. and Swamy, M. J. (2011) Thermodynamics of interaction of the major bovine seminal plasma protein, PDC-109 with phospholipid membranes. An isothermal titration calorimetric study. *PLoS ONE* 6, e25993.

Antony, T., Hoyer, W., Cherny, D., Heim, G., Jovin, T. M. and Subramaniam V. (2003) Cellular polyamines promote the aggregation of alpha synuclein. *J. Biol. Chem.* 278, 3235-3240.

Asthana, A., Raman, B., Ramakrishna, T., and Rao, C. M. (2012) Structural aspects and chaperone activity of human HspB3: Role of the ‘‘C-terminal extension.’’ *Cell Biochem. Biophys.* **64,** 61-72.

Atkin, K. E., Brentnall, A. S., Harris, G., Bingham, R. J., Erat, M. C., Millard, C. J., Linek, U. S., Staunton, D., Vakonakis, I., Campbell, I. D. and Potts, J. R. (2010) The Streptococcal Binding Site in the Gelatin-binding Domain of Fibronectin Is Consistent with a Non-linear Arrangement of Modules. . *J. Biol. Chem.* 285, 36977-36983.

Atmar, V. J., Kuehn, G. D. and Casillasg, E. R. (1981) A polyamine-dependent protein kinase from bovine epididymal spermatozoa. *J. Biol. Chem.* 256, 8275–8278.

Austin, C. R. (1951) Observations of the penetration of sperm into the mammalian egg. *Australian Journal of Scientific Research*, Series B, 4, 581-596.

Baker, M. E. (1985) The PDC-109 protein from bovine seminal plasma is similar to the gelatin-binding domain of bovine fibronectin and a kringle domain of human tissue-type plasminogen activator. *Biochem. Biophys. Res. Commun.* 130, 1010–1014.

Banyai, L., Tordai, H., and Patthy, L. (1994) The gelatin binding site of human 72 kDa type IV collagenase. *Biochem. J.* 298, 403-407.

Batra, J., Xu, K., Qin, S. And Zhou, H. X. (2009) Effect of Macromolecular Crowding on Protein Binding Stability: Modest Stabilization and Significant Biological Consequences. *Biophys. J.* 97, 906–911.

Bechor, D. S., and Levy, Y. (2008) Effect of glycosylation on protein folding: A close look at thermodynamic stabilization. *Proc. Natl. Acad. Sci. USA* 105, 8256-8261.

Benesch, J. L., Ayoub, M., Robinson, C. V. and Aquilina, J. A. (2008) Small heat shock protein activity is regulated by variable oligomeric substructure. *J. Biol. Chem.* 283:28513–28517.

Bergeron, J. J., Brenner, M. B, Thomas, D. Y., and Williams, D. B. (1994) Calnexin: a membrane-bound chaperone of the endoplasmic reticulum. *Trends Biochem. Sci.* 19, 124–128.

Bleil, J. D. and Wasserman, P. M. (1983) Sperm-egg interaction in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucid glycoprotein. *Dev. Biol. 95,* 317−324.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.

Briknarova, K., Grishaev, A., Banyai, L., Tordai, H., Patthy, L., and Llinas, M. (1999). The second type II module from human matrix metalloproteinase 2: Structure, function and dynamics. *Structure Folding Design*. 7, 1235–1245.

Brucker, C. and Lipford, G. B. (1995) The human sperm acrosome reaction: physiology and regulatory mechanisms. *Human Reproduction Update*. 1, 51-62.

Calvete, J. J., Raida, M., Sanz, L., Wempe, F., Scheit, K. H., Romer, A., and Töpfer-Petersen, E. (1994) Localization and structural characterization of an oligosaccharide O-linked to bovine PDC-109. Quantitation of the glycoprotein in seminal plasma and on the surface of ejaculated and capacitated spermatozoa. *FEBS Lett.* 350, 203–206.

Calvete, J. J., Varela, P. F., Sanz, L., Romero, A., Mann, K., and Töpfer-Petersen, E. (1996) A procedure for the large-scale isolation of major bovine seminal plasma proteins. *Protein Expr. Purif.* 8, 48–56.

Chandonnet, L., Roberts, K. D., Chapdelaine, A., and Manjunath, P. (1990) Identification of heparin-binding proteins in bovine seminal plasma. *Mol. Reprod. Dev.* 26, 313–318.

Chang, M. C. (1951) Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature*, 168, 697-698.

Chang, Z., Primm, T. P., Jakana, J., Lee, I. H., Serysheva, I., Chiu, W., Gilbert, H. F. and Quiocho F. A. (1996) Mycobacterium tuberculosis 16-kDa antigen (Hsp16.3) functions as an oligomeric structure in vitro to suppress thermal aggregation. *J. Biol. Chem.* 271, 7218–7223.

Charlton, L. M., Barnes, C. O., Li, Conggang, Orans, J., Young, G. B. and Pielak, G. J. (2008) Residue-Level Interrogation of Macromolecular Crowding Effects on Protein Stability. *J. Am. Chem. Soc.* 130, 6826–6830.

Chen, L., Drake, M. R., Resch, M. G., Greene, E. R., Himmel, M. E., Chaffey, P. K., Beckham, G. T. and Tan, Z. (2014) Specificity Of O-Glycosylation In Enhancing The Stability And Cellulose Binding Affinity Of Family 1 Carbohydrate-Binding Modules. *Proc. Natl. Acad. Sci. USA* 111, 7612-7617.

Chi, E. Y., Krishnan, S., Randolph, T. W. and Carpenter J. F. (2003) Physical Stability of Proteins in Aqueous Solution: Mechanism and Driving Forces in Nonnative Protein Aggregation. *Pharm. Res.* 9, 1325-1336.

Christiansen, A. and Stafshede, P. W. (2013) Quantification of Excluded Volume Effects on the Folding Landscape of *Pseudomonas aeruginosa* Apoazurin *In Vitro. Biophys J.* 105, 1689-1699.

Christiansen, A., Wang, Q., Cheung, M. S. and Wittung-Stafshede, P. (2013) Effects of macromolecular crowding agents on protein folding in vitro and in silico. *Biophys. Rev.* 5, 137–145.

Cohen, F. E. and Kelly, J. W. (2003) Therapeutic approaches to protein-misfolding diseases. *Nature.* 426, 905-909.

Constantine, K. L., Madrid, M., Banyai, L., Trexler, M., Patthy, L. and Llinas, M. (1992) Refind solution structure and ligand-binding properties of PDC-109 domain b. A collagen-binding type II domain. *J. Mol. Biol.* 223, 281-298.

Cornwell, D.G. and Moriski, N. (1984) Lipid peroxides and cell proliferation. In Pryor, W. (ed.), *Free Radicals in Biology.* Academic Press, New York, p. 119.

Creighton, T. E. (1992) Protein folding pathways determined using disulphide bonds. *Bioessays* 14, 195-199.

Damai, R. S., Sankhala, R. S., Anbazhagan, V., and Swamy, M. J. (2010) Destabilization of cell and model membranes by the major bovine seminal plasma protein, PDC-109: ^{31}P-NMR and AFM studies. *IUBMB Life* 62, 841-851.

Damai, R. S., Tarafdar, P. K., Singh, B. P., Reddy, S. T. and Swamy M. J. (2015) Biophysical characterization of the interaction of O-acylcholines with the major bovine seminal plasma protein, PDC-109. *Adv. Exp. Med. Biol.* 842, 279-292.

Das, K. P., Surewicz, W. K. (1995) Temperature-induced exposure of hydrophobic surfaces and its effect on the chaperone activity of alpha-crystallin. *FEBS Lett.* 369, 321-325.

Dean, D. C., Bowlus, C. L. and Bourgeois, S. (1987) Biochemistry Cloning and analysis of the promoter region of the human fibronectin gene *Proc. Natl. Acad. Sci. USA*. 84, 1876-1880.

Desnoyers, L. and Manjunath, P. (1992) Major proteins of bovine seminal plasma exhibit novel interactions with phospholipids. *J. Biol. Chem.* 267, 10149–10155.

Dhar, A., Samiotakis, A., Ebbinghaus, S., Nienhaus, L., Homouz, D., Gruebele, M. and Cheung, M. S. (2010). Structure, function, and folding of phosphoglycerate kinase are strongly perturbed by macromolecular crowding. *Proc. Natl. Acad. Sci. U S A*. 107, 17586-17591.

Eddy, E. M. and O'Brien, D. A. (1994) The Physiology of Reproduction. *Raven Press, New York.* 29-77.

Edstrom, A. M. L., Malm, J., Frohm, B., Martellini, J. A., Giwercman, A., Morgelin, M., Cole, A. M., and Sorensen, O. E. (2008) The major bactericidal activity of human seminal plasma is zinc-dependent and derived from fragmentation of the semenogelins. *J. Immunol.* 181, 3413-3421.

Ellis R. J. (2001) Macromolecular crowding: obvious but underappreciated. *Trends Biochem Sci*. 26, 597-604.

Ellis RJ. (2007) Protein misassembly: macromolecular crowding and molecular chaperones. *Adv. Exp. Med. Biol.* 594, 1–13.

Esch, F. S., Ling, N. C., Böhlen, P., Ying, S. Y. and R. Guillemin (1983). Primary structure of PDC-109, a major protein constituent of bovine seminal plasma, *Biochem. Biophys. Res. Commun*. 113, 861–867.

Finn, T. E., Nunez, A. C., Sunde, M. and Easterbrook-Smith S. B. (2012) Serum albumin prevents protein aggregation and amyloid formation and retains chaperone-like activity in the presence of physiological ligands. *J. Biol. Chem.* 287, 21530-21540

Flesch, F. M. and Gadella B. M. (2000) Dyamics of the mammalian sperm plasma membrane in the process of fertilization. *Biochim Biophys Acta.* 1469, 197-235.

Franzmann, T. M., Menhorn, P., Walter, S. and Buchner, J. (2008) Activation of the chaperone Hsp26 is controlled by the rearrangement of its thermosensor domain. *Mol. Cell.* 29, 207–216.

Gasset, M., Saiz, J. L., Sanz, L., Gentzel, M., Töpfer-Petersen, E., and Calvete, J. J. (1997) Conformational features and thermal stability of bovine seminal plasma protein PDC-109 oligomers and phosphorylcholine-bound complexes. *Eur. J. Biochem.* 250, 735–744.

Gennes, P. G. (1979) Scaling Concepts in Polymer Physics; Cornell University Press: Ithaca, NY

Gershenson, A. and Gierasch, L. M. (2011) Protein folding in the cell: challenges and progress. *Curr. Opin. Struct. Biol.* 21, 32–41.

Gnutt, D., Gao, M., Brylski, O., Heyden, M. and Ebbinghaus, S. (2015) Excluded-Volume Effects in Living Cells. *Angew Chem* (Int. Ed .Engl.) 54, 2548-2551.

Gonzalez, J. M., Jimenez, M., Velez, M., Mingorance, J., Andreu, J. M., Vicente, M. and Rivas, G. (2003) Essential cell division protein FtsZ assembles into one monomer-thick ribbons under conditions resembling the crowded intracellular environment. *J. Biol. Chem.* 278, 37664–37671.

Groen, J., Foschepoth, D., Brinke, E. T., Boersma, A. J., Imamura, H., Rivas, G., Heus, H. A. and Huck, W. T. S. (2015) Associative Interactions in Crowded Solutions of Biopolymers Counteract Depletion Effects. *J. Am. Chem. Soc.* 5b07898.

Gu, L., Abulimiti, A., Li, W. and Chang, Z. (2002) Monodisperse Hsp16.3 nonamer exhibits dynamic dissociation and reassociation, with the nonamer dissociation prerequisite for chaperone-like activity. *J. Mol. Biol.* 319, 517–526.

Gutsche, S., Von, W. M., Strowitzki, T. and Thaler, C. J. (2003) Seminal plasma induces mRNA expression of IL-1β, IL-6 and LIF in endometrial epithelial cells *in vitro*. *Mol Human Reprod* . 9, 785-791.

Gwathmey, T. M., Igmotz, G. G. and Suarez, S. S. (2003) PDC-109 (BSP-A1/A2) Promotes Bull Sperm Binding to Oviductal Epitelium In Vitro and May Be Involved in Forming the Oviductal Sperm Reservoir. *Biol. Reprod.* 69, 809-815.

Harada, Y., Ozaki, K., Suzuki, M., Fujiwara, T., Takahashi, E., Nakamura, Y., and Tanigami, A. (1999). Complete cDNA sequence and genomic organization of a human pancreas-specific gene homologous to Caenorhabditis elegans sel-1. *J. Hum. Genet*. 44, 330–336.

Harrison, R. A. P. (1996) Capacitation mechanisms, and the role of capacitation as seen in eutherian mammals. *Reprod. Fertil. Dev.* 8, 581–596.

Haslbeck, M., Walke, S., Stromer, T., Ehrnsperger, M., White, H. E., Chen, S., Saibil, H. R. and Buchner, J. (1999) Hsp26: a temperature-regulated chaperone. *EMBO J.* 18, 6744–6751.

Haslbeck, M., Franzmann, T., Weinfurtner, D. and Buchner, J (2005) Some like it hot: The structure and function of small heat-shock proteins. *Nat Struct Mol Biol* 12:842–846.

Haslbeck, M., Kastenmuller, A., Buchner, J., Weinkauf, S. and Braun, N. (2008) Structural dynamics of archaeal small heat shock proteins. *J. Mol. Biol.* 378, 362–374.

Hatters, D. M., Minton, A. P. and Howlett, G. J. (2002) Macromolecular crowding accelerates amyloid formation by human apolipoprotein C-II. *J. Biol. Chem.* 277, 7824–7830.

Hebert, D. N., and Molinari, M. (2007) In and out of the ER: protein folding, quality control, degradation, and related human diseases. *Physiol. Rev.* 87, 1377–1408

Hong, J. and Gierasch L. M., (2010) Macromolecular crowding remodels the energy landscape of a protein by favoring a more compact unfolded state. *J. Am. Chem. Soc.* 132, 10445-10452

Horwitz, J. (1992) Alpha-crystallin can function as a molecular chaperone. *Proc. Natl. Acad. Sci. USA.* 89, 10449-10453.

Huang, L. Q., Jin, R., Li, J. R., Luo, K., Huang, T., Wu, D., Wang, W. X., Chen, R. and Xiao, G. F. (2010) Macromolecular crowding converts the human recombinant PrPC to the soluble neurotoxic beta-oligomers. *FASEB J.* 24, 3536–3543

Huleihel, M., Lunenfeld, E., Levy, A., Potashnik, G. and Glezerman, M. (1996) Distinct expression levels of cytokines and soluble cytokine receptors in seminal plasma of fertile and infertile men. *Fertil Steril*. 66, 135-139.

Humphreys, D. T., Carver, J. A., Easterbrook-Smith, S. B., and Wilson M. R. (1999) Clusterin has chaperone-like activity similar to that of small heat shock proteins. *J. Biol. Chem.* 274, 6875-6881.

Igarashi, K. and Kashiwagi, K. (2010) Modulation of cellular function by polyamines. *Int. J. Biochem. Cell Biol.* 42, 39–51.

Ignotz, G., Lo, M. C., Perez, C. L., Gwathmey, T. M. and Suarez, S. S. (2001) Characterization of a fucose binding protein from bull sperm and seminal plasma that may be responsible for formation of oviductal sperm reservoir. *Biol. Reprod. 64*, 1806−1811.

Ikawa, M., Wada, I., Kominami, K., Watanabe, D., Toshimori, K., Nishimune,Y., and Okabe, M. (1997) The putative chaperone calmegin is required for sperm fertility. *Nature.* 387, 607– 611.

Jaya, N., Garcia, V. and Vierling, E. (2009) Substrate binding site flexibility of the small heat shock protein molecular chaperones. *Proc. Natl. Acad. Sci. USA.* 106, 15604–15609.

Juyena, N. S., and Stelletta, C. (2012) Seminal plasma: An essential attribute to spermatozoa. *J. Androl.* 33, 536–551.

Koteiche H. A. and Mchaourab, H. S. (2006) Mechanism of a hereditary cataract phenotype. Mutations in alpha-crystallin activate substrate binding. *J. Biol. Chem.* 281, 14273-14279.

Kumar, M. S., Reddy, P. Y., Sreedhar, B., and Reddy, G. B. (2005) αB-Crystallin assisted reactivation of glucose-6-phosphate dehydrogenase upon refolding. *Biochem. J.* 391, 335–341.

Lane, M. E., Thérien, I., Moreau, R., and Manjunath, P. (1999). Heparin and high-density lipoprotein mediate bovine sperm capacitation by different mechanisms. *Biol. Reprod.* 60, 169–175.

Larson, C. L., Samuelson, D. R., Eucker, T. P., Loughlin, J. L. and Konkel, M., E. (2013) The fibronectin-binding motif within FlpA facilitates *Campylobacter jejuni* adherence to host cell and activation of host cell signalling. *Emerging Microbes and Infections.* 2, e65

Lee, G. J., Roseman, A. M., Saibil, H. R. and Vierling, E. (1997) A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state. *EMBO J.* 16, 659–671.

Lefebvre, J., Fan, J., Chevalier, S., Sullivan, R., Carmona, E., and Manjunath, P. (2007) Genomic structure and tissue-specific expression of human and mouse genes encoding homologues of the major bovine seminal plasma proteins. *Mol. Hum. Reprod.* 13, 45-53.

Lefebvre, J., Boileau, G., and Manjunath, P. (2009) Recombinant expression and affinity purification of a novel epididymal human sperm-binding protein, BSPH1. *Mol. Hum. Reprod.* 15, 105–114.

Lenzi, A., Picardo, M., Gandini, L., and Dondero, F. (1996) Lipids of the sperm plasma membrane: from polyunsaturated fatty acids considered as markers of sperm function to possible scavenger therapay. *Hum. Reprod. Update* 3, 246-256.

Luo, J., Yu, C. H., Yu, H., Borstnar, R., Kamerlin, S. C. L., Slund, A. G., Abrahams, J. P. and Warmalander, S. K. T. S. (2013) Cellular polyamines promote amyloid-beta (Aβ) peptide fibrillation and modulate the aggregation pathways. *ACS Chem. Neurosci.* 4, 454–462.

Manjunath, P. and Sairam, M. R. (1987) Purification and biochemical characterization of three major acidic proteins (BSP-A1, BSP-A2 and BSP-A3) from bovine seminal plasma. *Biochem. J.* 241, 685–692.

Manjunath, P., Sairam, M. R. and Uma, J. (1987) Purification of four gelatin-binding proteins from bovine seminal plasma by affinity chromatography, *Biosci. Rep.* 7, 231–238.

Manjunath, P., Baillargeon, L., Marcel, Y. L., Seidah, N. G., Chretien, M., and Chapdelaine, A. (1988) Diversity of novel proteins in gonadal fluids. In Chretien, M. ed Molecular Biology of Brain and Endocrine Peptidergic Systems *New York Springer International Publishing AG, Biochemical Endocrinology* pp 259-273.

Manjunath, P., Nauc, V., Bergeron, A., and Menard, M. (2002) Major proteins of bovine seminal plasma bind to the low density lipoprotein fraction of hen's egg yolk. *Biol. Reprod.* 67, 1250–1258.

Mann, T. (1954) The Biochemistry of Semen. *John Wiley & Sons, New York,* 223pp.

Mann, T. (1964) The Biochemistry of Semen and of the Male Reproductive Tract. *Methuen, London.* 493-507

Mann, T. (1981) Male Reproductive Function and Semen. *Springer-Verlag, Berlin.* 495-512

Martinez, H. R., Kvist, U., Ernerudh, J., Sanz, L. And Calvete, J. J. (2011). Seminal Plasma Proteins: What Role Do They Play? *American Journal of Reproductive Immunology*. 66, 11-22

Mattews, C. H. and Van Holde, K. E. (eds) (1990) Lipid metabolism 2°. In *Biochemistry*. *Benjamins-Cummins*, 613–615.

Mendoza, J. A., Rogers, E., Lorimer, G. H. and Horowitz, P. M. (1991) Chaperonins facilitate the in vitro folding of monomeric mitochondrial rhodanese. *J. Biol. Chem.* 266, 13044-13049.

Miklos, A. C., Li, Conggang, Sharaf, N. G. and Pielak G. J. (2010) Volume Exclusion and Soft Interaction Effects on Protein Stability under Crowded Conditions. Biochemistry 49, 6984-6991.

Miklos, A. C., Sarkar, M., Wang, Y. and Pielak, G. J. (2011) Protein crowding tunes protein stability. *J. Am. Chem. Soc.* 133, 7116–7120.

Miller, D., Brough, S., and al-Harbi, O. (1992) Characterization and cellular distribution of human spermatozoal heat shock proteins. *Hum. Reprod.* 7, 637–645.

Minton, A. P. and Ellis R. J. (2003) Cell biology: Join the crowd. *Nature*. 425, 27-28.

Minton, A. P. (2005a) Models for excluded volume interaction between an unfolded protein and rigid macromolecular cosolutes: macromolecular crowding and protein stability revisited. *Biophys. J.* 88, 971-85.

Minton AP. (2005b) Influence of macromolecular crowding upon the stability and state of association of proteins: Predictions and observations. *Journal of Pharmaceutical Sciences*. 94, 1668–1675.

Mitra, N., Sinha, S., Ramya, T. N. C. and Surolia, A. (2006) N-linked oligosaccharides as outfitters for glycoprotein folding, form and function. *Trends. Biochem. Sci.* 31, 156-163.

Momo, F., Wisniewska, A. and Stevanato, R. (1995) EPR study of multilamellar phosphatidylcholine liposomes. *Biochim. Biophys. Acta* 1240, 89–94.

Moremen, K. W., Tiemeyer, M., and Alison, V. N. (2012) Vertebrate protein glycosylation: diversity, synthesis and function. *Nat. Rev. Mol. Cell Biol.* 13, 448–462.

Mosher, D. F. (1993). Assembly of fibronectin into extracellular matrix. *Curr. Opin. Struct. Biol.* 3, 214–222.

Müller, P., Erlemann, K. R., Müller, K., Calvete, J. J., Töpfer-Petersen, E., Marienfield, K., and Herrmann, A. (1998) Biophysical characterization of the interaction of the bovine seminal plasma protein PDC-109 with phospholipid vesicles. *Eur. Biophys. J.* 27, 33-41.

Mukherjee, S., Waegele, M. M, Chowdhury, P., Guo. L. and Gai, F. (2009) Effect of macromolecular crowding on protein folding dynamics at the secondary structure level. *J. Mol. Biol.* 393, 227-236.

Munishkina, L. A., Cooper, E. M., Uversky, V. N. and Fink, A. L. (2004) The effect of macromolecular crowding on protein aggregation and amyloid fibril formation. *J. Mol. Recognit.* 17, 456-464.

Murray, L. S., Lu, Y., Taggart, A., Van Regemorter, N., Vilain, C., Abramowicz, M., Kadler, K. E. and Van Agtmael, T. (2014) Chemical chaperone treatment reduces intracellular accumulation of mutant collagen IV and ameliorates the cellular phenotype of a COL4A2 mutation that causes haemorrhagic stroke. *Hum. Mol. Genet.* 23, 283-292.

Napper, C. E., Drickamer, K., and Taylor, M. E. (2006) Collagen binding by the mannose receptor mediated through the fibronectin type II domain. *Biochem. J.* 395, 579-586.

Nowotarski, S.L., Woster, P. M. and Casero, R. A. Jr. (2013) Polyamines and cancer: implications for chemotherapy and chemoprevention. *Expert Rev. Mol. Med.* 15, e3.

Özcan, U., Yilmaz, E., Özcan, L., Furuhashi, M., Vaillancourt, E., Smith R. O., Görgün, C., Z. and Hotamisligil. G. S. (2006) Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science.* 313, 1137-1140

Ozhogina, O. A., Trexler, M., Banyai, L., Llinas, M., and Patthy, L. (2001) Origin of fibronectin type II (FN2) modules: Structural analyses of distantly-related members of the kringle family identify the kringle domain of neurotrypsin as a potential link between FN2 domains and kringles. *Prot. Sci.* 10, 2114-2122.

Parekh, R. B. (1991) Effects of glycosylation on protein function. *Curr. Opin. Struct. Biol.* 1, 750-754.

Pegg, A. E. (2014) The function of spermine. *IUBMB Life.* 66, 8–18.

Patthy, L., Trexler, M., Vali, Z., Banyai, L. and Varadi, A. (1984) Kringles: Modules specialized for protein binding. Homology of the gelatine-binding region of fibronectin with kringle structure of proteases. *FEBS Letters*. 171, 131-136.

Petro, G. D., Barlati, S., Vartio, T. and Vaheri A. (1981) Transformation-enhancing activity of gelatine-binding fragments of fibronectin. *Proc. Natl. Acad. Sci. USA.* 78, 4965-4969.

Pickford, A. R., Potts, J. R., Bright, J. R., Phan, I. and Campbell, I. D. (1997) Solution structure of a type 2 module from fibronectin: implications for the structure and function of the gelatin binding domain. *Structure* 5, 359-370.

Politch, J.A., Tucker, L., Bowman, F. P., and anderson D. J. (2007) Concentrations and significance of cytokines and other immunologic factors in semen of healthy fertile men. *Human Reprod* . 22, 2928-2935.

Ramakrishnan, M., Anbazhagan, V., Pratap, T. V., Marsh, D. and Swamy, M. J. (2001) Membrane insertion and lipid-protein interactions of bovine seminal plasma protein PDC-109 investigated by spin label electron spin resonance spectroscopy. *Biophys. J.* 81, 2215–2225.

Raman, B., Ramakrishna, T. and Rao., C. M. (1996) Refolding of Denatured and Denatured/Reduced Lysozyme at High Concentrations. *J. Biol. Chem.* 271, 17067-17072.

Raman, B., Rao, C. M. (1994) Chaperone-like activity and quaternary structure of alpha-crystallin. *J. Biol. Chem.* 269, 27264-27268.

Ruan, H., Hill, J. R., Fatemie-Nainie, S. and Morris, D. R. (1994) Cell-specific translational Regulation of S-adenosylmethionine decarboxylase mRNA. Influence of the structure of the 5′ transcript leader on regulation by the upstream open reading frame. *J. Biol. Chem.* 269, 17905–17910.

Rubinstein, S., Lax, Y., Shalev, Y., and Breitbart, H. (1995) Dual effect of spermine on acrosomal exocytosis in capacitated bovine spermatozoa. *Biochim. Biophys. Acta.* 1266, 196–200.

Rubinstein, S. and Breitbart, H. (1991) Role of spermine in mammalian sperm capacitation and acrosome reaction. *Biochem. J.* 278, 25–28.

Saling, P. M. (1991) How the egg regulates sperm function during gamete interaction: facts and fantasies. *Biol. Reprod.*, 44, 246–251.

Sankhala, R. S., Damai, R. S., Anbazhagan, V., Kumar, C. S., Bulusu, G., and Swamy, M. J. (2011) Biophysical investigations on the interaction of the major bovine seminal plasma protein, PDC-109 with heparin. *J. Phys. Chem. B.* 115, 12954-12962.

Sankhala, R. S., and Swamy M. J., (2010) The major protein of bovine seminal plasma, PDC-109, is a molecular chaperone. *Biochemistry* 49, 3908–3918.

Sankhala, R. S., Damai, R. S. and Swamy, M. J. (2011) Correlation of membrane binding and hydrophobicity to the chaperone-like activity of PDC-109. *PLoS ONE* 6, e17330.

Sankhala, R. S., Kumar, C. S., Singh, B. P., Arangasamy, A., and Swamy, M. J. (2012) HSP-1/2, a major protein of equine seminal plasma, exhibits chaperone-like activity. *Biochem. Biophys. Res. Commun.* 427, 18-23.

Sawkar, A. R., Cheng, W. C., Beutler, E., Wong, C. H., Balch, W. E. and Kelly, J. W. (2002). Chemical chaperones increase the cellular activity of N370S beta-glucosidase: a therapeutic strategy for Gaucher disease. *Proc. Natl. Acad. Sci. U.S.A.* 99, 15428-15433.

Scheit, K. H., Kemme, M., Aumüller, G., Seitz, J., Hagendorff, G. and Zimmer, M. (1988) The major protein of bull seminal plasma: biosynthesis and biological function. *Biosci. Rep.* 8, 589–608.

Schellman, J. A. (1975) Macromolecular binding. *Biopolymers* 14, 999−1018.

Seidah, N. G., Manjunath, P., Rochemont, J., Sairam, M. R. and Chere´tian, M. (1987) Complete amino acid sequence of BSP-A3 from bovine seminal plasma. Homology to PDC-109 and to the collagen-binding domain of fibronectin, *Biochem. J.* 243, 195–203.

Sharma, K. K., Kaur, H., Kumar, G. S. and Kester, K. (1998) Interaction of 1,1_-bi(4-anilino) naphthalene-5,5-disulfonic acid with alpha-crystallin. *J. Biol. Chem.* 273, 8965–8970.

Shashidharamurthy, R., Koteiche H. A., Dong, J., McHaourab, H. S. (2005) Mechanism of chaperone function in small heat shock proteins: Dissociation of the HSP27 oligomer is required for recognition and binding of destabilized T4 lysozyme. *J. Biol. Chem.* 280, 5281–5289.

Shiraki, K., Kudou, M., Aso, Y. and Takagi, M. (2003) Dissolution of protein aggregation by small amine compounds. *Sci. Technol. Adv. Mat.* 4, 55–59.

Singh, D., Raman, B., Ramakrishna, T. and Rao C. M. (2006) The cataract-causing mutation G98R in human alphaA-crystallin leads to folding defects and loss of chaperone activity. *Mol. Vis.* 12, 1372-1379.

Skorstengaard, K., Holtet, T. L., Etzerodt, M. and Thogersen, H. C. (1994) Collagen-binding recombinant fibronectin fragments containing type II domains. *FEBS Lett.* 343, 47-50.

Snoussi, K. and Halle, B. (2005) Protein self-association induced by macromolecular crowding: a quantitative analysis by magnetic relaxation dispersion. *Biophys. J.* 88, 2855–2866.

Soucek, K., Slabakova, E., Ovesna, P., Malenowska, A., Kozubik, A. And Hampl A (2010). Growth/differentiation factor-15 is an abundant cytokine in human seminal plasma. *Human Reprod* 25, 2962-2971.

Spiro, R. G. (2002) Protein glycosylation: nature, distribution, enzymatic formation, and disease implication of glycopeptides bonds. *Glycobiology* 12, 43R-56R.

Stewart, E. M., Aquilina, J. A., Easterbrook-Smith, S. B., Danielle Murphy-Durland, D., Jacobsen, C., Moestrup, S., and Wilson, M. R. (2007) Effects of glycosylation on the structure and function of the extracellular chaperone clusterin. *Biochemistry* 46, 1412-1422.

Sticht, H., Pickford, A. R., Potts, J. R., and Campbell, D. (1998) Solution structure of the glycosylated second type 2 module of fibronectin. *J. Mol. Biol.* 276, 177-187.

Sultan, A., Raman, B., Rao, Ch. M., and Tangirala, R. (2013) The extracellular chaperone haptoglobin prevents serum fatty acid-promoted amyloid fibril formation of β2-microglobulin, resistance to lysosomal degradation, and cytotoxicity. *J. Biol. Chem.* 288, 32326-32342.

Swamy, M. J. (2004) Interaction of bovine seminal plasma proteins with model membranes and sperm plasma membranes. *Curr. Sci. 87*, 203−211.

Tannert, A., Kurz, A., Erlemann, K. R., Müller, K., Herrmann, A., Schiller, J., Töpfer-Petersen, E., Manjunath, P., Müller, P. (2007) The bovine seminal plasma protein PDC-109 extracts phosphorylcholine-containing lipids from the outer membrane leaflet. *Eur. Biophys. J.* 36, 461-75.

Therien, I., Bleau, S. G. and Manjunath, P. (1995) Phosphatidylcholine-binding proteins of bovine seminal plasma modulate capacitation of spermatozoa by heparin. *Biol. Reprod.* 52, 1372-1379.

Thérien, I., Moreau, R., and Manjunath, P. (1998). Major proteins of bovine seminal plasma and high-density lipoprotein induce cholesterol efflux from epididymal sperm. *Biol. Reprod*. 59, 768–776.

TÖpfer-petersen, E., Ekhlasi-Hudrieser, M., Kirchhoff, C., Leeb, T. and Sieme, H. (2005) The role of stallion seminal plasma proteins in fertilisation. *Anim Reprod Sci.* 89, 159-170

Toshimori , K. (2009) Dynamics of the Mammalian Sperm Head. *Springer-Verlag, Berlin.* 31-39

Uversky, V. N., Cooper, E. M., Bower, K. S., Li, J. and Fink, A. L. (2002) Accelerated alpha-synuclein fibrillation in crowded milieu. *FEBS Lett.* 515, 99–103.

Van, M. R. L., Basha, E., Friedrich, K. L., Slingsby, C. and Vierling, E. (2001) Crystal structure and assembly of a eukaryotic small heat shock protein. *Nat. Struct. Biol.* 8, 1025–1030.

van Montfort, R. L., Basha, E., Friedrich, K. L., Slingsby, C. & Vierling, E. Crystal structure and assembly of a eukaryotic small heat shock protein. *Nat. Struct. Biol.* 8, 1025–1030.

Vik, A., Aas, F. E., Anonsen, J. H., Bilsborugh, S., Schneider, A., Jacobsen, W. E., and Koomey, A. (2008) Broad spectrum O-linked protein glycosylation in the human pathogen *Neisseria gonorrhoeae*. *Proc. Natl. Acad. Sci. USA.* 106, 4447-4452.

Visconti, P. E., Calantino-Hormer, H., Moore, G. D., Bailey, J. L., Ning, X., Fornes, M. and Kopf, G. S. (1998) The molecular basis of sperm capacitation. *J. Androl.* 19, 242–248.

Wah, D. A., Tornero, C. F., Sanz, L., Romero, A., and Calvete, J. J., (2002) Sperm Coating Mechanism from the 1.8Å Crystal Structure of PDC-109-Phosphorylcholine Complex, *Structure*. 10, 505–514.

Ward, C. R. and Kopf, G. S. (1993) Molecular events mediating sperm activation. *Dev. Biol. 104,* 287−296.

Wassarman, P. M. (1990a) Profile of a mammalian sperm receptor. *Development.* 108, 1–17.

Wassarman, P. M. (1990b) Regulation of mammalian fertilization by zona pellucida glycoproteins. *J. Reprod. Fertil.*, 42, 79–87.

Wassarman, P. M. (1999) Mammalian fertilization: Molecular aspects of gamete adhesion, exocytosis and fusion. *Cell.* 96, 175–183.

White, D. A., Buell, A. K., Knowles, T. P. J., Welland, M. E. And Dobson, C. M. (2010) Protein Aggregation in Crowded Environments. *J. Am. Chem. Soc.* 132, 5170–5175.

Williams, K. (1997) Interactions of polyamines with ion channels. *Biochem. J.* 325, 289–297.

Wilson, M. R. (2011) Clusterin facilitates *in vivo* clearance of extracellular misfolded proteins. *Cell. Mol. Life Sci.* 68, 3919–3931.

Wyatt, A. R., Yerbury, J. J., Ecroyd, H., and Wilson M. R. (2013) Extracellular chaperones and proteostasis. *Annu. Rev. Biochem.* 82, 295–322.

Yanagimachi, R. (1994) Mammalian fertilization. In the Physiology of Reproduction, E. Knobil and J. Neill. *Raven Press, New York.* 189-317.

Zimmerman, S. B. and Trach, S. O. (1988) Effects of macromolecular crowding on the association of E. coli ribosomal particles. *Nucleic Acids Res.* 16, 6309–6326.

Zimmerman, S. B. and Minton, A. P. (1993) Macromolecular crowding: biochemical, biophysical, and physiological consequences. *Annu. Rev. Biophys. Biomol. Struct.* 22, 27–65.

Zhou, H. X., Rivas, G. and Minton A. P. (2008) Macromolecular crowding and confinement: biochemical, biophysical, and potential physiological consequences. *Annu. Rev. Biophys.* 37, 375-397.

Zhou, H. X. (2004) Polymer models of protein stability, folding and interactions. *Biochemistry.* 43, 2141-2154.

Zhou H. X. (2008) Protein folding in confined and crowded environments. *Arch. Biochem. Biophys.* 469, 76-82.

Zhou, H. X. (2013) Influence of crowded cellular environments on protein folding, binding, and oligomerization: Biological consequences and potentials of atomistic modeling. *FEBS Lett.* 587, 1053-1061.

Zorrilla, S., Rivas, G., Acuna, A. U. and Lillo, M. P. (2004) Protein self-association in crowded protein solutions: A time-resolved fluorescence polarization study. *Protein Science.* 13, 2960–2969.

Zwaal, R. F. A., and Schroit, A. J. (1997) Pathophysiologic implication of membrane phospholipid asymmetry in blood cells. *Blood* 89, 1121-1132

Publications

1. Anzabazhagan, V., Sankhala, R. S., **Singh, B. P.,** Swamy, M. J. (2011), Isothermal Titration Calorimetric studies of the major bovine seminal plasma protein, PDC-109 with phospholipid membrane. ***PLoS One***, 6(10) e25993

2. Sankhala, R.S., Kumar, C. S., **Singh B. P.,** Arangasamy, A., Swamy, M. J. (2012) HSP-1/2, a major protein of equine seminal plasma, exhibits chaperone-like activity. ***Biochem Biophys Res Commun***. 427, 18-23.

3. Damai, R. S., Tarafdar, P. K., **Singh, B. P.,** Reddy, S. T., Swamy, M. J. (2015) Biophysical characterization of the interaction of O-acylcholine with the major protein of bovine seminal plasma, PDC-109. ***Adv. Exp. Med. Biol***. 842, 279-292

4. **Singh, B. P.,** Sankhala, R. S., Asthana, A., Basu, A., Murthi, T. R., Rao, Ch. M. and Swamy, M. J. Mutational Analysis of the Highly Conserved Core Tryptophan Residues in the Fibronectin Type-II Domains of the Major Protein of Bovine Seminal Plasma, PDC-109. (Manuscript under preparation)

5. **Singh, B. P.,** Saha, I. and Swamy, M. J. Effect of Polyamines on Chaperone Like Activity and Lipid Binding Ability of Bovine Seminal Plasma Protein PDC-109. (Manuscript under preparation)

6. **Singh, B. P.,** Saha, I. and Swamy, M., J. Molecular crowding differentially affects the chaperone-like and lipid binding activities of PDC-109. (Manuscript under preparation)