THE JOURNAL OF PHYSICAL CHEMISTRY B

ARTICLE

pubs.acs.org/JPCB

Biophysical Investigations on the Interaction of the Major Bovine Seminal Plasma Protein, PDC-109, with Heparin

Rajeshwer S. Sankhala,[†,∥] Rajani S. Damai,[†,∥] V. Anbazhagan,[†] C. Sudheer Kumar,[†] Gopalakrishnan Bulusu,[‡,§] and Musti J. Swamy*[,†]

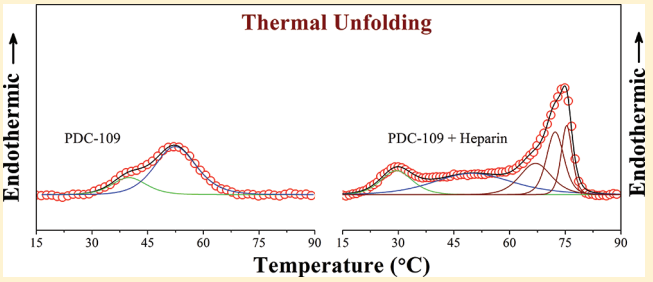
[†]School of Chemistry, University of Hyderabad, Hyderabad 500 046, India

[‡]International Institute of Information Technology, Hyderabad 500032, India

[§]TCS Innovation Laboratories, Tata Consultancy Services, Hyderabad 500081, India

Supporting Information

ABSTRACT: PDC-109, the major bovine seminal plasma protein, binds to sperm plasma membrane and modulates capacitation in the presence of heparin. In view of this, the PDC-109/heparin interaction has been investigated employing various biophysical approaches. Isothermal titration calorimetric studies yielded the association constant and changes in enthalpy and entropy for the interaction at 25 °C (pH 7.4) as 1.92 (±0.2) × 10^5 M^{-1}, 18.6 (±1.6) kcal M^{-1}, and 86.5 (±5.1) cal M^{-1} K^{-1}, respectively, whereas differential scanning calorimetric studies indicated that heparin binding results in a significant increase in the thermal stability of PDC-109. The affinity decreases with increase in pH and ionic strength, consistent with the involvement of electrostatic forces in this interaction. Circular dichroism spectroscopic studies indicated that PDC-109 retains its conformational features even up to 70−75 °C in the presence of heparin, whereas the native protein unfolds at about 55 °C. Atomic force microscopic studies demonstrated that large oligomeric structures are formed upon binding of PDC-109 to heparin, indicating an increase in the local density of the protein, which may be relevant to the ability of heparin to potentiate PDC-109 induced sperm capacitation.



INTRODUCTION

In mammals fertilization takes place when spermatozoa from the male interact with the egg in the female uterus and fuses with it. Various studies show that sperm cells do not possess the ability to fertilize the egg at the time of ejaculation, but acquire it during their transit through the female genital tract by a process termed capacitation.[1] Despite its discovery over 50 years ago,[2,3] the detailed steps involved in capacitation are not yet clearly understood.

Studies on different species have shown that the seminal plasma contains proteinaceous factors that play important roles in priming the sperm cells for fertilization. In the bull (*Boa taurus*), the seminal plasma contains a group of four closely related acidic proteins—called BSP-A1, BSP-A2, BSP-A3, and BSP-30-kDa—that bind to sperm plasma membranes upon ejaculation by specific interaction with the phospholipids.[4] Together these proteins are referred to as bovine seminal plasma proteins (BSP proteins). BSP-A1 and BSP-A2 are identical in their amino acid sequence and differ only in the degree of glycosylation. A mixture of these two proteins, referred to as PDC-109, constitutes the major protein fraction of the bovine seminal plasma and is present at a concentration of 15−25 mg/mL.[5] PDC-109 contains two tandemly repeating fibronectin type-II (FnII) domains, each of which can bind to a choline phospholipid on the sperm plasma membrane by its specific interaction with the phosphorylcholine headgroup.[6] This interaction of PDC-109 with the sperm cell membrane results in an efflux of cholesterol and choline phospholipids, a process referred to as cholesterol efflux, which appears to be important for capacitation.[7,8] In view of this, the interaction of PDC-109 with different phospholipid membranes has been investigated using a variety of biophysical approaches.[9−19] Very recent studies show that PDC-109 also exhibits chaperone like activity against a variety of target proteins in vitro, which increases significantly in the presence of phosphatidylcholine and phosphatidylcholine/cholesterol mixtures.[20,21]

Besides binding to the phospholipid membranes, PDC-109 also interacts with a variety of other ligands including apolipoproteins A1 and A2, several types of collagens, and heparin.[22−25] Heparin and other glycosaminoglycans (GAGs) are components of follicular and oviductal fluids, which play an important role in sperm capacitation.[26,27] The interaction of BSP proteins with heparin has been exploited in their purification by affinity chromatography and it has been demonstrated that while

Received: April 15, 2011
Revised: September 21, 2011
Published: September 22, 2011

polydisperse PDC-109 binds heparin, the monomeric form does not display heparin binding ability.[22,28] In addition, proteolytic digestion and chemical modification studies demonstrated that lysine and arginine residues are important for heparin binding, indicating that the PDC-109/heparin association is mediated predominantly by electrostatic interactions.[28]

It is known that preincubation with heparin increases the fertilizing ability of bovine spermatozoa.[29,30] Preincubation of spermatozoa with BSP proteins has also been reported to increase acrosome reaction,[31] whereas obstruction of heparin binding to the BSP proteins with specific antibodies decreases it significantly.[27] Taken together, these studies suggest that BSP/heparin interactions are important for the sperm capacitation process, although a molecular level understanding of the role of heparin in BSP induced sperm capacitation remains unclear. It is therefore important to investigate the interaction of PDC-109 and other BSP proteins with heparin. In the present study, the energetics and mechanism of PDC-109 binding to heparin have been investigated using isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC), which have been used widely to characterize sugar−protein interactions.[32−34] ITC studies indicate the presence of multiple binding sites on heparin for PDC-109. The PDC-109/heparin interaction is associated with a close compensation of enthalpy and entropy, although the latter appears to be a dominant driving force for the interaction. Change in pH and ionic strength strongly affect the PDC-109/heparin interaction, with the affinity decreasing at higher pH and ionic strength. Analysis of DSC data shed further light on the interacting species, binding affinities, and thermal stability of PDC-109, upon its interaction with heparin. Both ITC and DSC results indicate the formation of oligomeric structures upon PDC-109/heparin interaction, which was further confirmed by atomic force microscopy (AFM). The ability of a single heparin molecule to bind multiple protein molecules indicates that heparin binding increases the local density of the protein, which could be of significance during sperm capacitation. CD studies are in good agreement with DSC results and suggest that heparin binding stabilizes the structure of PDC-109. Biophysical investigations using ITC, fluorescence spectroscopy, and circular dichroism indicate that both polydisperse and dimeric PDC-109 binds to phosphorylcholine (PrC), whereas only the polydisperse form can interact with heparin, which is consistent with earlier reports.[28] The present observations are of significant interest in view of the crucial role played by heparin in sperm capacitation and acrosome reaction.

MATERIALS AND METHODS

Materials. Phosphorylcholine chloride (Ca^{2+} salt), Tris base, and heparin (sodium salt, M_r 3000) were obtained from Sigma (St. Louis, MO). Heparin (M_r 6000) was purchased from Sisco Research Laboratory (Mumbai, India). Sephadex G-50 (superfine) and DEAE Sephadex A-25 were purchased from Pharmacia (Uppsala, Sweden). All other chemicals used were of analytical grade and were obtained from local suppliers. PDC-109 was purified from bovine seminal plasma from healthy and reproductively active bulls by gel filtration and affinity chromatography as reported earlier.[11,35]

Isothermal Titration Calorimetry. PDC-109 was dialyzed extensively against Tris buffer (pH 7.4) containing 50 mM Tris, 150 mM NaCl, 5 mM EDTA, and 0.025% sodium azide (TBS-I) and diluted to a final working concentration of 0.20−0.25 mM. Calorimetric titrations were performed using a VP-ITC microcalorimeter from MicroCal (Northampton, MA). The solutions were degassed under vacuum before using in order to prevent bubbling. The data were acquired and analyzed using the Origin software supplied by MicroCal. Binding of heparin to PDC-109 was investigated at various temperatures as follows. Typically, 20−40 aliquots of 1 mM heparin (5 μL each) were injected successively from the syringe into the calorimeter cell of 1.445 mL filled with the protein solution. Successive additions were separated by 200 s intervals to allow the endothermic peak resulting from the reaction to return to the baseline. To ensure proper mixing after each injection, a constant stirring speed of 300 rpm was maintained during the experiment. To minimize the contribution to the binding heat from dilution, the protein and heparin solutions were prepared in the same buffer. Control experiments were performed by titrating heparin solution into TBS-I, and the resulting heat changes were subtracted from the measured heats of binding. The first injection is often inaccurate; therefore, a 2 μL injection was added first and the resultant data point was deleted before analyzing the remaining data.

Effect of pH and Ionic Strength on the Interaction of PDC-109 with Heparin. PDC-109 was taken in sufficiently high concentration and dialyzed against buffers of the desired pH. Tris buffer (50 mM) containing 150 mM NaCl and 5 mM EDTA at different pH (7.4−9.0) was used as the buffering system. ITC experiments were performed essentially as described above by injecting heparin (1 mM) from the syringe to the calorimeter cell containing ca. 200 μM of PDC-109 at 25 °C. In order to probe the effect of ionic strength of the medium on the PDC-109/heparin interaction, two different samples of the protein were freshly dialyzed against 50 mM tris buffer (pH 7.4) and TBS-I, respectively at 4 °C. The protein was then diluted with the appropriate buffer to yield a final concentration of 200 μM. Heparin (1 mM) was also dissolved in the above buffers and titrations were performed at 20 °C.

Differential Scanning Calorimetry. DSC measurements were performed on a MicroCal VP DSC apparatus (MicroCal LLC, Northampton, MA, USA). PDC-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 22.9 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 heparin (M_r 3 kDa) binding on the thermal stability of PDC-109 were carried out under similar conditions and the data obtained were analyzed using the "non-two-state mode of fitting" model in the MicroCal Origin software. The binding affinity of heparin to PDC-109, at the thermal transition point, was analyzed according to eq 1:[36]

$$K_b(T_c) = [\exp\{(T_c - T_m)\Delta H_c/nRT_cT_m\} - 1]/[L] \quad (1)$$

where $K_b(T_c)$ is the binding constant at the reference temperature, ΔH_c is the calorimetric enthalpy of the PDC-109/heparin complex, T_c is the denaturation temperature in the presence of heparin, T_m is the denaturation temperature in the absence of heparin, R is the universal gas constant, [L] is the ligand concentration, and n is the number of binding sites on the protein.

Atomic Force Microscopy. Appropriate aliquots drawn from a freshly dialyzed sample of PDC-109 (1 mg/mL) in TBS-I and a 5 mM solution of heparin in the same buffer were mixed to yield a 1.0 mL solution containing 0.4 mg/mL of the protein and 0.5 mM heparin. After a 30-min incubation, a 20 μL aliquot

from the above mixture was deposited on a freshly cleaved mica sheet and allowed to bind for ~30 min, the unbound material was washed off with HPLC grade distilled water, and samples were transferred to the AFM stage for imaging, essentially as described earlier.[20] PDC-109 and heparin (independently) at the concentrations indicated above were used as controls.

Circular Dichroism Spectroscopy. CD spectral studies were performed using a JASCO J-810 spectropolarimeter fitted with a thermostatted cell holder and a thermostatic water-bath. Spectra were recorded using a 0.2 cm path length quartz cell. The effect of temperature on the conformation of PDC-109 was probed in two different ways. First, PDC-109 was preincubated with a fixed concentration of heparin and ellipticities were recorded as a function of wavelength at fixed temperatures between 30 and 80 °C. Concentrations of PDC-109 and heparin used for the far- and near-UV spectra are mentioned in the respective figure captions. Each spectrum reported was the average of 20 consecutive scans from which buffer scans, recorded under the same conditions, were subtracted. The observed ellipticities were converted to mean residue ellipticities (MRE).

In the second type of experiments, PDC-109 was preincubated with different concentrations of heparin and ellipticities were recorded at fixed wavelengths (221 nm in the far UV region and 289 nm in the near UV region) as a function of temperature. The temperature was increased from 25 to 90 °C at a scan rate of 1°/min. To investigate the effect of heparin binding on the conformational stability of the protein, PDC-109 was incubated with a fixed concentration of heparin, before recording the CD spectra.

Effect of PrC/PDC-109 Interaction on Heparin Binding. The effect of PrC binding to PDC-109 on its interaction with heparin was investigated using ITC and fluorescence spectroscopy. For ITC experiments PDC-109, preincubated with 20 mM PrC (resultant concentration), was taken in the calorimeter cell, to which were added small aliquots of heparin from the syringe. The experiment was carried out at 25 °C, and a constant stirring speed of 300 rpm was maintained during the experiment.

Steady state fluorescence measurements were performed using a Spex model Fluoromax-3 spectrofluorimeter at room temperature (~25 °C), with both the excitation and emission bandpass filters set at 3 nm. To a PDC-109 solution (OD_{280} = 0.1) in TBS-I were added small aliquots of PrC from a 50 mM stock solution. Upon saturation, small aliquots of heparin (from a 0.5 mM stock solution) were added, in order to investigate if heparin could dissociate the PDC-109/PrC complex. Experiments were also performed by reversing the sequence of the addition of PrC and heparin. Samples were excited at 280 nm and emission spectra were recorded between 320 and 400 nm. All spectra were corrected for dilution and inner filter effects.

RESULTS AND DISCUSSION

Thermodynamics of PDC-109 Binding to Heparin. Thermodynamic parameters characterizing the interaction of PDC-109 with heparin were obtained from ITC studies. Figure 1 gives an ITC profile for the binding of PDC-109 to heparin at 20 °C. From this figure it is seen that addition of heparin to PDC-109 solution yields large endothermic heats of binding. The magnitude of the endothermic peaks decreases with subsequent injections, showing saturation of the binding sites at high heparin concentrations. The data could be best fitted by a nonlinear least-squares method to the "one set of sites" binding model among

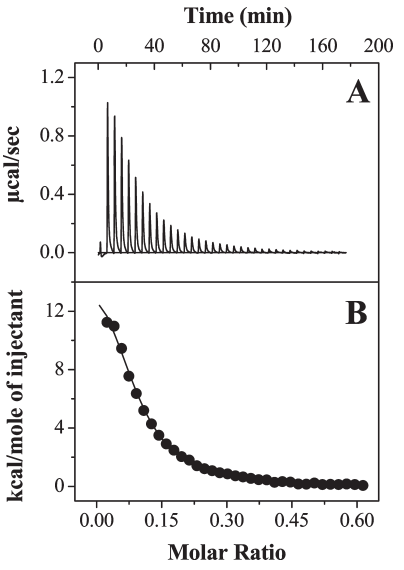


Figure 1. Isothermal titration calorimetry of heparin binding to PDC-109. (A) Raw data for the titration of 0.25 mM PDC-109 with 1 mM heparin (M_r ~6000) at 20 °C. (B) Integrated heats of binding obtained from the raw data shown in panel A, after subtracting the heat of dilution. The solid line represents the best curve fit to the experimental data, using the "one set of sites model" from MicroCal Origin.

the various models available in the Origin ITC data analysis software. The binding constant (K_b), stoichiometry (n), enthalpy of binding (ΔH), entropy of binding (ΔS), and free energy of binding (ΔG) characterizing the PDC-109/heparin interaction at various temperatures, obtained from this analysis, are listed in Table 1.

The results presented in Table 1 indicate that the PDC-109/heparin interaction is stabilized by entropy of binding, with a negative enthalpic contribution. It is also seen from Table 1 that the binding enthalpy increases in magnitude with temperature; however, the entropy of binding increases more steeply resulting in a net increase in the free energy of binding, as reflected by an increase in the binding affinity. The positive value of ΔS for the binding of PDC-109 to heparin indicates that during the binding process counterions bound to the charged groups as well as water molecules that are closely associated with the protein and heparin are expelled from the interface of PDC-109/heparin complex. Values of the stoichiometry (n), which corresponds to the number of PDC-109 molecules that interact with each molecule of heparin, are found to range between 7.9 and 18.2 mol of PDC-109 per mole of heparin (M_r ~6 kDa). The above result indicates the presence of multiple binding sites on heparin for the protein molecules, which is consistent with the results obtained with other heparin binding proteins such as antithrombin III, heparin binding growth-associated molecule, HIV-1 TAT protein etc.[34,37,38]

Since PDC-109 exists as a polydisperse aggregate in the absence of choline containing ligands,[39] it would be difficult to get a precise estimate of the number of sites on the heparin molecule for PDC-109. The average molecular weight of heparin used in this study is ~6 kDa, indicating that each heparin molecule on average contains about 20 monosaccharide units. Therefore it is possible that each molecule of heparin can in principle offer a number of binding sites for interaction with PDC-109. This was further confirmed by using a lower molecular

Table 1. Thermodynamic Parameters for the Binding of PDC-109 to Heparin (M_r ~6000) at Different Temperatures[a]

T (°C)	n[b]	$K_a \times 10^{-4}$ (M^{-1})	$-\Delta G$ (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1})
14	18.2 (±2.4)	5.4 (±1.1)	6.09	12.1 (±2.0)	63.4 (±7.3)
18	12.8 (±0.5)	10.5 (±1.4)	6.69	17.7 (±1.1)	83.6 (±4.0)
20	7.9 (±1.6)	12.8 (±1.2)	6.85	16.0 (±0.8)	78.0 (±2.9)
25	11.0 (±0.4)	19.2 (±0.2)	7.20	18.6 (±1.6)	86.5 (±5.1)
30	12.8 (±0.3)	46.2 (±1.3)	7.85	33.8 (±0.1)	137.5 (±0.5)

[a] Values given correspond to the averages obtained from two or three independent titrations and standard deviations are indicated in parentheses. [b] n = number of moles of PDC-109 per mole of heparin.

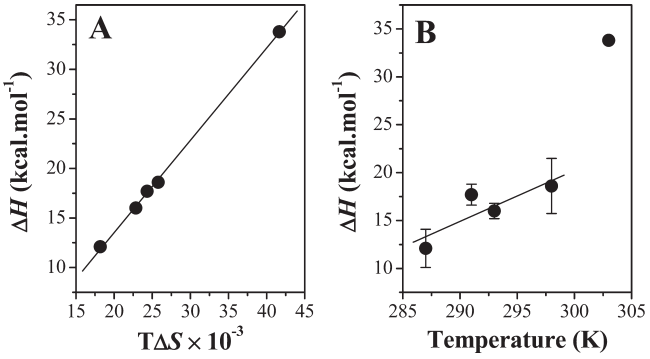


Figure 2. ITC studies on heparin binding to PDC-109. (A) Enthalpy–entropy compensation plot for the interaction of PDC-109 with heparin. The straight line corresponds to a linear least-squares fit. (B) Temperature dependence of the reaction enthalpies of PDC-109 upon binding to heparin. From the slope of the linear least-squares fit, the ΔC_p value was obtained.

weight heparin (~3 kDa) for the binding titrations. The results obtained demonstrate that only 3–4 molecules of PDC-109 bind to a single molecule of heparin (M_r 3 kDa), which is considerably less than the stoichiometry obtained above for the binding of PDC-109 to heparin (M_r 6 kDa) under similar experimental conditions. This indicates that the larger heparin molecule presents more binding sites for interaction with PDC-109. The value of n was observed to be sensitive to the temperature change and this could be due to changes in the aggregation state of PDC-109 with temperature.

In order to visualize the interaction of multiple PDC-109 molecules with a single molecule of heparin, we performed molecular modeling studies using the known 3-dimensional structures of PDC-109 (PDB ID: 1H8P) and heparin decamer (PDB ID: 3OFL) (see the Supporting Information for details). The rudimentary molecular dynamics simulations performed here indicate that K34, R64, K68, and K59 are proximal for interaction with heparin (Figure S1). This is in good agreement with the results obtained from chemical modification studies,[7] which implicated 6 basic residues, namely K34, R57, K59, R64, K68, and R104, in the interaction of PDC-109 with heparin. Interestingly, when a second protein molecule was docked to the PDC-109/heparin complex, in some of the docked structures obtained the second protein molecule was found to associate with the first molecule of PDC-109 rather than with heparin (Figure S2). Our results indicate that about 4–6 molecules of PDC-109 can directly bind to a heparin molecule containing 20 monosaccharide units (see Figure S3). This shows that the experimentally observed stoichiometry of 7.9–18.2 protein molecules per heparin (M_r ~6 kDa) can be achieved only if oligomeric species of PDC-109 bind to heparin wherein some of the monomers cannot directly interact with the heparin.

An enthalpy–entropy compensation plot (ΔH versus $T\Delta S$) for the binding of heparin to PDC-109 is shown in Figure 2A. The linearity of the data presented in this figure suggests that binding of heparin to PDC-109 is associated with close compensation between enthalpy and entropy. An exact compensation of enthalpy by entropy would be expected to yield a slope close to 1.0 and deviation from this value would indicate the predominance of one of these factors over the other. For slope >1.0, binding is governed primarily by enthalpy, whereas a slope <1.0 indicates a process that it is predominantly entropy-driven. The observed slope of 0.931 (±0.015) for the fit in Figure 2A is consistent with the above interpretation that binding of heparin to PDC-109 is driven predominantly by entropic forces. A plot of the binding enthalpy versus temperature for the PDC-109/heparin interaction is shown in Figure 2B. It is seen that the ΔH values for this interaction exhibit a linear dependence from 14 to 25 °C. The slope obtained by analyzing the data presented in Figure 2B to a linear least-squares fit yielded the heat capacity change (ΔC_p) value as 530 (±238) cal mol^{-1} K^{-1} for the binding of heparin to PDC-109. The large positive heat capacity is not unusual and has been reported earlier for the interaction of heparin sulfate with transduction domain of HIV-1 TAT protein.[34] Usually positive enthalpy of binding is associated with hydrophobic interactions. However, both classical and nonclassical hydrophobic effects are characterized by a negative heat capacity change,[40] and therefore the above result is not consistent with hydrophobic interactions playing a major role in the PDC-109/heparin association. On the contrary, charge neutralization is accompanied by a positive ΔC_p and the binding reaction may proceed even if ΔH is substantially endothermic because of the positive ΔS.[34] Hence the present ITC results suggest that electrostatic interactions play a significant role in the binding of heparin with PDC-109, which is consistent with earlier reports based on chemical modification of the protein.[28]

Effect of pH and Ionic Strength on the Interaction of PDC-109 with Heparin. The effect of pH and ionic strength on the thermodynamic parameters associated with the interaction of PDC-109 with heparin was studied using ITC, and the values of various parameters obtained are listed in Table S1. Measurements were performed only in the narrow pH range of 7.4–9.0 since the protein tended to precipitate at low pH and heparin does not show any observable affinity for the protein at pH 9.0 and above. ITC profiles for the interaction of heparin with PDC-109 at different pH are shown in Figure 3. It is clear from Figure 3A that at pH 7.4 heparin completely saturates all of the binding sites on PDC-109 and binding heat approaches the baseline, whereas with increasing pH fewer binding sites are saturated by heparin (Figure 3, panels B and C), indicating decreased affinity of heparin

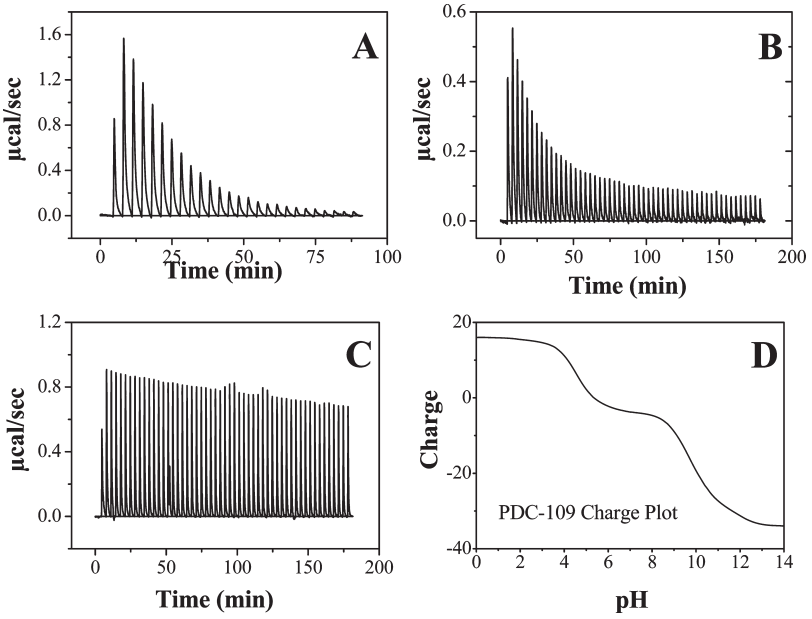


Figure 3. Effect of pH on heparin binding to PDC-109. ITC profiles for the titration of 0.2 mM PDC-109 with 1 mM heparin at pH 7.4 (A), 8.0 (B), and 9.0 (C). T = 25 °C. For all of the experiments, 50 mM tris buffer containing 150 mM NaCl and 5 mM EDTA was used and the pH was adjusted to the appropriate value with HCl. (D) pH dependence of the surface charge of PDC-109, obtained using the "protein charge analysis" tool of CLCbio (www.clcbio.com).

with increase in the pH. The results presented in Table S1 are in good agreement with the above interpretation and show that the enthalpy, entropy and affinity of heparin binding to PDC-109 decrease with increasing pH and no more binding is observed at pH ≥9.0. The binding of heparin to PDC-109 has been reported to involve primarily electrostatic interactions.[28] Heparin is a highly sulfated glycosaminoglycan and bears a large negative charge density, whereas PDC-109 is an acidic protein with an isoelectric point of ∼5.0. When pH of the medium is increased, PDC-109 becomes more negatively charged, which is also clear from the surface charge plot shown in Figure 3D. At high pH both PDC-109 and heparin will be highly negatively charged and due to the electrostatic repulsion between them the binding is expected to be significantly weakened, if not completely abrogated, indicating that the interaction is predominantly mediated by electrostatic forces. ITC experiments aimed at investigating the effect of ionic strength of the medium on the PDC-109/heparin interaction yielded a binding affinity of 1.14×10^6 M^{-1} in 50 mM tris buffer (Figure S4), which decreased to 1.28×10^5 M^{-1} upon inclusion of 150 mM NaCl in the buffer (Table S1). This decrease in the affinity further confirms the electrostatic nature of the PDC-109/heparin interaction.

Thermal Denaturation of PDC-109 in the Absence and Presence of Heparin: DSC Studies. The thermal stability of PDC-109 in the presence of different concentrations of heparin was studied using high-sensitivity DSC. Thermograms corresponding to PDC-109 alone and in the presence of various concentrations of heparin, corrected for buffer baseline are shown in Figure 4. The data shown in these thermograms could be best fitted to a to a non-two-state model. Native PDC-109 exhibits two thermotropic transitions centered at about 313 and 327 K, which were attributed to the dissociation of oligomeric protein (into monomers) and unfolding of the monomer, respectively.[39] Analysis of the calorimetric scans obtained in the presence of heparin required a minimum of five components that undergo thermal unfolding at different temperatures to obtain a satisfactory fit of the resolved components to the experimentally obtained thermograms. A representative example displaying the resolved components corresponding to the thermogram obtained in the presence of 0.5 mM heparin is shown in Figure 4A. Values of the calorimetric enthalpy to van't Hoff enthalpy ratio ($\Delta H_c/\Delta H_v$) corresponding to these transitions are given in Table 2, which sheds light on the oligomeric status of the protein.[41] The $\Delta H_c/\Delta H_v$ ratios for the first transition (in the presence of varying concentrations of heparin) was observed to be in the range of 0.15−0.39, indicating the presence of small oligomeric aggregates. The transition temperature for the dissociation of these oligomeric species was observed to be about 303−304 K, which matches with the temperature at which the PDC-109 oligomers dissociate.[39] For the second transition, the $\Delta H_c/\Delta H_v$ ratios were in the neighborhood of 1 and the transition temperatures were around 324−327 K, which are consistent with the unfolding of PDC-109 monomers.[39] The $\Delta H_c/\Delta H_v$ ratios for remaining transitions (3rd−5th) were observed to be in the range of 0.03−0.21 (Table 2), indicating the presence of large oligomeric structures. Since multiple PDC-109 monomers can bind to one molecule of heparin (Table 1), these oligomers, to a large extent, must consist of PDC-109/heparin complexes. The transition temperatures observed for such oligomeric states are in the range of 336−350 K (Table 2), indicating that PDC-109/heparin complexes form stable structures, which maintain their integrity even at higher temperatures. Total enthalpy change (ΔH_T) for PDC-109 in the presence of heparin was calculated by adding the enthalpy corresponding to each component. The total enthalpy change obtained from the DSC study for PDC-109 alone was 45 kcal.mol^{-1}, whereas the total enthalpy values estimated from the DSC scans performed in the presence of different concentrations of heparin ranged between 76.8 and 97.0 kcal mol^{-1} (see Figure S5). Binding of PrC was also reported to result in a significant increase in the enthalpy of

unfolding of PDC-109 along with an increase in the unfolding temperature.[39] The increase in ΔH_T with increasing heparin concentration indicates that heparin binding stabilizes the structure of PDC-109, which was further confirmed by CD spectroscopy (see below).

The binding affinities between PDC-109 and heparin at denaturing temperatures were calculated using Schellman's equation[36] and the data obtained are shown in Table S2. Since this method does not provide accurate values at lower ligand concentrations,[42] the binding affinities were calculated at 1 and 0.5 mM concentration of heparin only. The observed affinities are significantly lower than those measured by calorimetric titrations performed between 14 and 30 °C (see Table 1), indicating that the affinity of interaction decreases at higher temperatures.

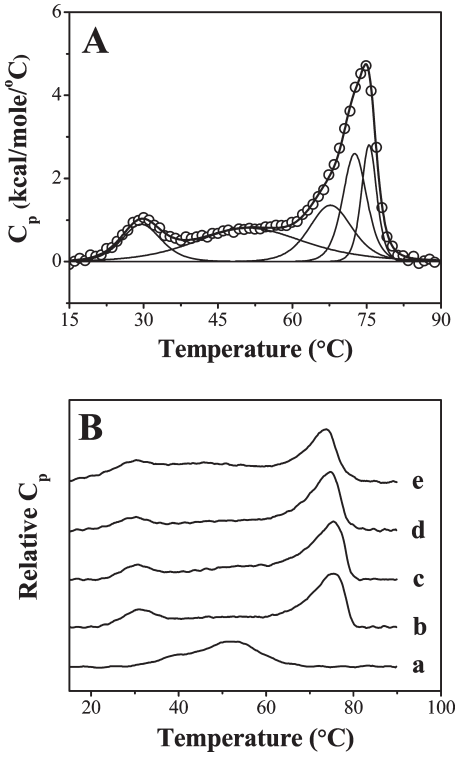


Figure 4. DSC studies on the effect of heparin binding on the thermal stability of PDC-109. (A) DSC thermogram of PDC-109 (0.6 mg/mL) in the presence of 0.5 mM heparin is shown as open circles (O) and the best fit of DSC data to the non-two-state transition model is shown as a solid thick line (—). The data could be fitted assuming 5 transitions as shown by thin solid (−) lines. (B) Calorimetric profiles of 0.6 mg/mL PDC-109 in TBS-I in the presence of increasing concentrations of heparin: 0 (a), 0.05 (b), 0.1 (c), 0.5 (d), and 1 mM (e). See text for details.

Heparin Induced Oligomerization of PDC-109. AFM images of PDC-109 in the absence and presence of heparin are shown in Figure 5. Being a polydisperse protein, native PDC-109 shows an average size distribution in the range of 1−4 nm, although a few oligomeric structures ranging from 20 to 150 nm can be observed at various places (Figure 5, panels A and B). PDC-109 which was preincubated with heparin showed an average size distribution in the range of 4−10 nm with frequent occurrence of larger oligomeric structures (50−300 nm; Figure 5, panels C and D). These observations suggest that binding of heparin to PDC-109 results in the formation of larger oligomeric structures, which is in good agreement with the results of ITC and DSC studies, discussed above.

Heparin Binding Increases the Thermal Stability of PDC-109. Heparin has previously been shown to stabilize protein and peptide conformers in solution and to impart thermal and chemical stability to peptide/heparin complexes.[32,43] The present DSC results clearly suggest that heparin stabilizes PDC-109 against thermal unfolding (cf. Figure S5). In order to probe the effect of heparin binding on the conformation of PDC-109 and monitor the effect of temperature on PDC-109/heparin interaction, we recorded CD spectra of PDC-109 in the absence and in the presence of heparin at various temperatures (Figures 6 and S6). The near-UV CD spectrum of PDC-109 is characterized by a broad positive band with two discrete maxima at 282 and 289 nm,

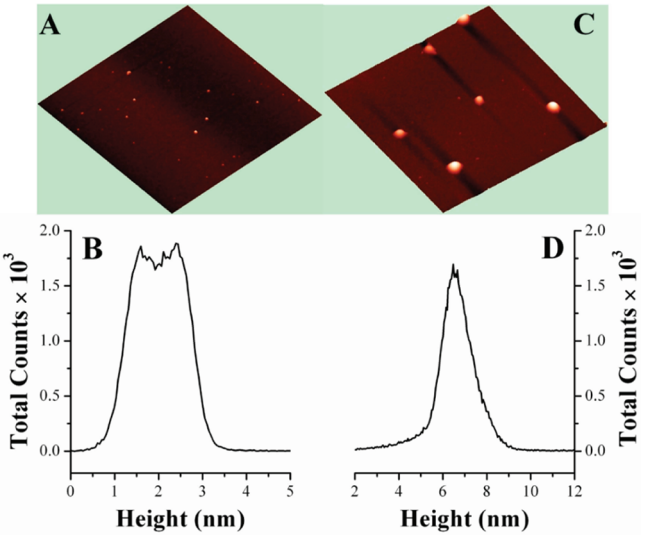


Figure 5. AFM studies on PDC-109/heparin interaction. (A) AFM image of native PDC-109 in air (5 × 5 μm). (B) Histogram corresponding to the image shown in panel A. (C) AFM image of PDC-109 upon interaction with heparin (5 × 5 μm). (D) Histogram corresponding to the image shown in panel C.

Table 2. Thermodynamic Parameters Obtained from DSC Measurements on the Thermal Transitions of PDC-109 in the Presence and Absence of Heparin[a]

[heparin] (mM)	T_{m1} (K)	$\Delta H_{c1}/\Delta H_{v1}$	T_{m2} (K)	$\Delta H_{c2}/\Delta H_{v2}$	T_{m3} (K)	$\Delta H_{c3}/\Delta H_{v3}$	T_{m4} (K)	$\Delta H_{c4}/\Delta H_{v4}$	T_{m5} (K)	$\Delta H_{c5}/\Delta H_{v5}$
0	313.0	0.40	326.5	0.8						
0.05	304.4 ± 0.1	0.15 ± 0.03	324.8 ± 0.1	0.8 ± 0.03	342.1 ± 0.1	0.19 ± 0.01	347.0 ± 0.1	0.098 ± 0.002	349.9 ± 0.1	0.029 ± 0.001
0.1	304.1 ± 0.4	0.16 ± 0.03	326.7 ± 0.1	0.88 ± 0.02	341.2 ± 0.1	0.2 ± 0.01	346.5 ± 0.1	0.1 ± 0.01	349.5 ± 0.1	0.041 ± 0.003
0.5	302.7 ± 0.1	0.18 ± 0.04	324.2 ± 0.7	0.98 ± 0.14	340.5 ± 0.3	0.21 ± 0.02	345.6 ± 0.1	0.11 ± 0.005	348.5 ± 0.1	0.045 ± 0.005
1.0	302.5 ± 0.2	0.39 ± 0.05	320.4 ± 0.5	1.07 ± 0.01	336.2 ± 1.1	0.19 ± 0.03	343.6 ± 0.4	0.18 ± 0.05	347.1 ± 0.1	0.08 ± 0.02

[a] Average values from three independent measurements are given along with the estimated standard deviations.

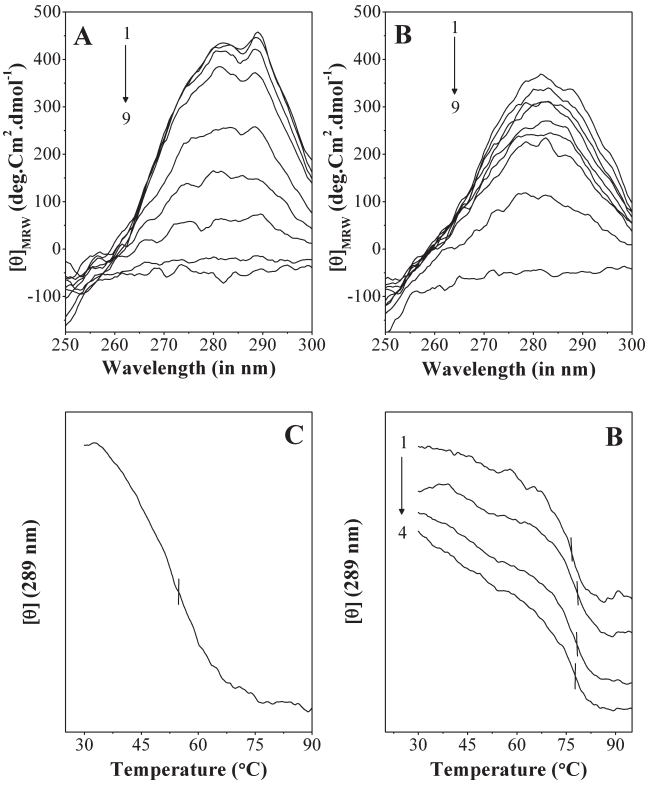


Figure 6. Effect of heparin binding on the thermal stability of PDC-109. Near-UV CD spectra of PDC-109 (1.0 mg/mL) at various temperatures in the absence (A) and presence (B) of 1 mM heparin. Arrow represents the direction of increase in the temperature. The temperatures are: (1) 25, (2) 30, (3) 35, (4) 40, (5) 50, (6) 55, (7) 60, (8) 70, and (9) 80 °C. Thermal scans of the protein (1.0 mg/mL) in the absence and presence of varying concentrations of heparin, at a fixed wavelength (289 nm, near-UV) are shown in panel (C) and (D). The concentrations of heparin are: (1) 0.03, (2) 0.05, (3) 0.2, and (4) 0.5 mM.

whereas the far-UV spectrum is characterized by a major positive band with a maximum at 223 nm and a shoulder at about 215 nm, consistent with previous reports.[16,39] Upon heparin binding the intensity of the near- and far-UV CD spectra decreased considerably (Figures 6B and S6B), indicating heparin induced conformational changes in the protein structure. Both the near and far-UV spectra of the free protein undergo a reduction in overall intensity as the temperature is increased up to 50 °C. Above 50 °C significant loss was observed in the secondary and tertiary structure of the protein and above 60 °C no dichroic behavior was observed with the native protein (Figures 6A and S6A), whereas significant amounts of both secondary and tertiary structures could be seen even at 70 °C in the presence of heparin (Figures S6B and 6B). The protein denatured rapidly above 70 °C.

In order to investigate the relation between the thermal stability of PDC-109 and heparin concentration, CD spectral intensities were recorded at a fixed wavelength corresponding to the maximum intensity as a function of temperature. The temperature dependence of CD signal intensity at 289 (near-UV) and 221 nm (far-UV), gave sigmoidal curves centered at ~55 °C, indicating that the midpoint of the unfolding transition of the protein is at ~55 °C (Figures 6C and S6C). With increasing concentrations of heparin the transition midpoint for the unfolding of PDC-109 shifted toward higher temperatures (70−78 °C; Figures 6D and S6D). Plots of changes in the transition midpoints of far- and near-UV spectral intensities against heparin concentration (Figures S7A and B), clearly showed that heparin stabilizes both the secondary and tertiary structures of PDC-109 in a concentration dependent manner.

Phosphorylcholine Inhibits PDC-109/Heparin Interaction. The effect of phosphorycholine binding to PDC-109 on its interaction with heparin was investigated by ITC and fluorescence spectroscopy (Figure 7). In the ITC experiments addition of small aliquots of heparin to the PDC-109 solution containing 20 mM PrC yielded negligible changes in the reaction heat (panels A and B), suggesting very weak or negligible binding of heparin to PDC-109. It has been reported that heparin binds to the oligomeric form of PDC-109 and dissociation of the oligomers either by high salt concentration or PrC binding results in a dramatic decrease in the PDC-109/heparin interaction.[28] The present ITC results are consistent with these observations.

Fluorescence studies aimed at investigating the effect of PrC binding on the interaction of heparin with PDC-109 are shown in Figure 7, panels C and D. Addition of PrC to PDC-109 results in a gradual increase in the fluorescence intensity initially, which tends to saturate with subsequent injections. An increase of approximately 19% was observed in the fluorescence intensity of PDC-109 upon addition of PrC (2.5 mM final concentration) and subsequent addition of heparin resulted in only ~2.3% decrease of the fluorescence intensity (panel C), suggesting that heparin does not bind to PDC-109/PrC complex. On the contrary when heparin was added first to PDC-109, it resulted in a net decrease of ~11% in the fluorescence intensity, which increased by ~17% upon subsequent addition of PrC (panel D). These observations can be explained as follows. Since PDC-109 exists as a polydisperse aggregate under natural conditions,[39] in the above experiment heparin can bind to the native protein which results in a net decrease in the fluorescence intensity. However, addition of PrC to the PDC-109/heparin mixture, results in a dissociation of the oligomeric protein into smaller aggregates (predominantly dimers). Since these aggregates have a very low affinity for heparin,[28] the dissociation of PDC-109 oligomers results in the dissociation of its complex with heparin, which in turn leads to enhanced fluorescence intensity. This was further supported by CD studies (Figures S8A and S8B), where preincubation of PDC-109 with PrC followed by the addition of heparin yielded a spectrum (dash dot line), which is similar to that observed in the presence of only PrC (dash line), indicating the association of PDC-109 with PrC rather than with heparin.

A Working Model. Our ITC and DSC results demonstrate that several molecules of PDC-109 can simultaneously bind to a single molecule of heparin, resulting in the formation of large oligomeric structures. This indicates that binding to heparin increases the local density of PDC-109, which is in good agreement with the results obtained with other heparin binding proteins.[34,37,38] Increased local density of protein should enhance its functional activity and this is consistent with the fact that sperm incubated in vitro with heparin display higher fertilizing capacity than sperm incubated without heparin.[29−31] In the female genital tract when seminal plasma comes in contact with oviductal fluid, PDC-109 interacts with heparin with a subsequent increase in its local concentration, which can capacitate the sperm in a more effective manner than native PDC-109. Indeed, Thérien et al.[31] have shown that spermatozoa preincubated with PDC-109 (BSP-A1/A2) alone show ~5% acrosome reaction, whereas preincubation of spermatozoa with a mixture

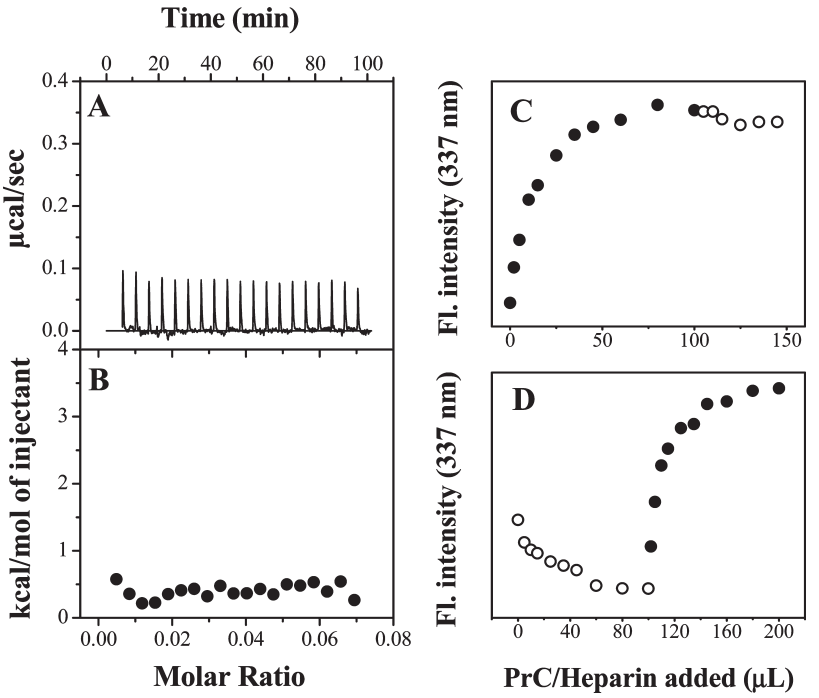


Figure 7. Effect of PrC binding on the PDC-109/heparin interaction. (A) Raw ITC data for the titration of 0.25 mM PDC-109 containing 20 mM PrC with 7 μL aliquots of 1 mM heparin. (B) Integrated heats of binding obtained from the raw data shown in the upper panel. (C) Fluorescence titration of PDC-109 with PrC followed by heparin. To PDC-109 was added PrC from a 50 mM stock solution in small aliquots (●), followed by addition of small aliquots of 0.5 mM heparin (○). (D) Fluorescence titration of PDC-109 with heparin followed by PrC. To PDC-109 was added heparin from a 0.5 mM stock solution in small aliquots (○), followed by addition of small aliquots of 50 mM PrC (●). See text for details.

of PDC-109 and heparin resulted in a significant increase in the acrosome reaction to ~25%, which is consistent with the above model.

CONCLUSIONS

The energetics of interaction of PDC-109 with heparin has been investigated by isothermal titration calorimetry and differential scanning calorimetry. ITC results suggest that 8−16 molecules of PDC-109 can bind to a single molecule of heparin and this interaction is predominantly driven by a large positive entropic contribution. Positive heat capacity change and decrease in PDC-109/heparin affinity with increasing pH/ionic strength indicate that PDC-109/heparin interaction is governed by electrostatic forces. Results of DSC studies suggest the presence of at least three types of PDC-109/heparin oligomeric clusters, which maintain their integrity even at elevated temperatures (>70 °C). Both DSC and CD studies suggest that the structure of PDC-109 is stabilized by heparin in a concentration dependent manner. Our results also strengthen the earlier report that heparin prefers oligomeric PDC-109 for binding rather than monomeric or dimeric forms. Overall, the present results explore the energetics of the PDC-109/heparin interaction and shed light on the molecular level details of this interaction, which plays a vital role in sperm capacitation.

ASSOCIATED CONTENT

Supporting Information. Parts of Methods and Results corresponding to molecular docking and molecular dynamics simulations and CD spectral studies, two tables (S1, S2), and eight figures (S1−S8). This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel: +91-40-2313-4807. Fax: +91-40-2301-2460. E-mail: mjssc@uohyd.ernet.in; mjswamy1@gmail.com. Website: http://202.41.85.161/~mjs/.

Author Contributions

‖These two authors contributed equally to this study.

ACKNOWLEDGMENT

This work was supported by a research project from the Department of Science and Technology (India) to M.J.S. R.S.S., R.S.D., and V.A. were supported by Senior Research Fellowships, and C.S.K. is supported by a Junior Research Fellowship from the CSIR (India). The MicroCal VP-ITC equipment used in this study was obtained from the funds made available to the University of Hyderabad through the UPE program of the University Grants Commission (India). We acknowledge the University Grants Commission (India) for their support through the CAS program to the School of Chemistry. We thank Meenakshi Pradhan (TCS Innovation Laboratories) for help with modelling and simulation studies.

REFERENCES

(1) Shivaji, S.; Scheit, K.-H.; Bhargava, P. M. *Proteins of the seminal plasma*; Wiley: New York, 1990; p 526.
(2) Chang, M. C. *Nature* **1951**, *168*, 697–698.

(3) Austin, C. R. *Nature* **1952**, *170*, 326.
(4) Desnoyers, L.; Manjunath, P. *J. Biol. Chem.* **1992**, *267*, 10149–10155.
(5) Scheit, K.-H.; Kemme, M.; Aümuller, G.; Seitz, J.; Hagendorff, G.; Zimmer, M. *Biosci. Rep.* **1988**, *8*, 589–608.
(6) Wah, D. A.; Fernández-Tornero, C.; Sanz, L.; Romero, A.; Calvete, J. J. *Structure* **2002**, *10*, 505–514.
(7) Thérien, I.; Moreau, R.; Manjunath, P. *Biol. Reprod.* **1998**, *59*, 768–776.
(8) Moreau, R.; Thérien, I.; Lazure, C.; Manjunath, P. *Biochem. Biophys. Res. Commun.* **1998**, *246*, 148–154.
(9) Müller, P.; Erlemann, K.-R.; Müller, K.; Calvete, J. J.; Töpfer-Petersen, E.; Marienfeld, K.; Herrmann, A. *Eur. Biophys. J.* **1998**, *27*, 33–41.
(10) Gasset, M.; Magdaleno, M.; Calvete, J. J. *Arch. Biochem. Biophys.* **2000**, *250*, 735–744.
(11) Ramakrishnan, M.; Anbazhagan, V.; Pratap, T. V.; Marsh, D.; Swamy, M. J. *Biophys. J.* **2001**, *81*, 2215–2225.
(12) Greube, A.; Müller, K.; Töpfer-Petersen, E.; Herrmann, A.; Müller, P. *Biochemistry* **2001**, *40*, 8326–8334.
(13) Swamy, M. J.; Marsh, D; Anbazhagan, V; Ramakrishnan, M. *FEBS Lett.* **2002**, *528*, 230–234.
(14) Thomas, C. J.; Anbazhagan, V.; Ramakrishnan, M.; Sultan, N.; Surolia, I.; Swamy, M. J. *Biophys. J.* **2003**, *84*, 3037–3044.
(15) Swamy, M. J. *Curr. Sci.* **2004**, *87*, 203–211.
(16) Anbazhagan, V.; Swamy, M. J. *FEBS Lett.* **2005**, *579*, 2933–2938.
(17) Anbazhagan, V.; Damai, R. S.; Paul, A.; Swamy, M. J. *Biochim. Biophys. Acta* **2008**, *1784*, 891–899.
(18) Damai, R. S.; Anbazhagan, V.; Rao, K. B.; Swamy, M. J. *Biochim. Biophys. Acta* **2009**, *1794*, 1725–1733.
(19) Damai, R. S.; Sankhala, R. S.; Anbazhagan, V.; Swamy, M. J. *IUBMB Life* **2010**, *62*, 841–851.
(20) Sankhala, R. S.; Swamy, M. J. *Biochemistry* **2010**, *49*, 3908–3918.
(21) Sankhala, R. S.; Damai, R. S.; Swamy, M. J. *PLoS ONE* **2011**, *6*, e17330.
(22) Manjunath, P.; Sairam, M. R.; Uma, J. *Biosci. Rep.* **1987**, *7*, 231–238.
(23) Chandonnet, L.; Roberts, K. D.; Chapdelaine, A.; Manjunath, P. *Mol. Reprod. Dev.* **1990**, *26*, 313–318.
(24) Manjunath, P.; Nauc, V.; Bergeron, A.; Menard, M. *Biol. Reprod.* **2002**, *67*, 1250–1258.
(25) Manjunath, P.; Marcel, Y. L.; Uma, J.; Seidah, N. G.; Cheretian, M.; Chapdelaine, A. *J. Biol. Chem.* **1989**, *264*, 16853–16857.
(26) Parrish, J. J.; Susko-Parrish, J. L.; Handrow, R. R.; Ax, R. L.; First, N. L. *Gamete Res.* **1989**, *24*, 403–413.
(27) Lane, M.-E.; Thérien, I.; Moreau, R.; Manjunath, P. *Biol. Reprod.* **1999**, *60*, 169–175.
(28) Calvete, J. J.; Campanero-Rhodes, M. A.; Raida, M.; Sanz, L. *FEBS Lett.* **1999**, *444*, 260–264.
(29) Parrish, J. J.; Susko-Parrish, J. L.; First, N. L. *Theriogenology* **1985**, *24*, 537–549.
(30) Parrish, J. J.; Susko-Parrish, J. L.; Leibfried-Rutledge, M. L.; Critser, E. S.; Eyestone, W. H.; First, N. L. *Theriogenology* **1986**, *25*, 591–600.
(31) Thérien, I.; Bleau, G; Manjunath, P. *Biol. Reprod.* **1995**, *52*, 1372–1379.
(32) Evans, D. L.; Marshall, C. J.; Christey, P. B.; Carrell, P. W. *Biochemistry* **1992**, *31*, 12629–12642.
(33) Surolia, A.; Sharon, N.; Schwarz., F. P. *J. Biol. Chem.* **1996**, *271*, 17697–17703.
(34) Ziegler, A.; Seelig, J. *Biophys. J.* **2004**, *86*, 254–263.
(35) Calvete, J. J.; Varela, P. F.; Sanz, L.; Romero, A.; Mann, K.; Töpfer-Petersen, E. *Protein Expr. Purif.* **1996**, *8*, 48–56.
(36) Schellman, J. A. *Biopolymers* **1975**, *14*, 999–1018.
(37) Tyler-Cross, R.; Sobel, M.; Marques, D.; Harris, R. B. *Protein Sci.* **1994**, *3*, 620–627.
(38) Fath, M.; VanderNoot, V.; Kilpeläinen, I.; Kinnunen, T.; Rauvala, H.; Linhardt, R. J. *FEBS Lett.* **1999**, *454*, 105–108.
(39) Gasset, M.; Saiz, J. L.; Laynez, J.; Sanz, L.; Gentzel, M.; Töpfer-Petersen, E.; Calvete, J. J. *Eur. J. Biochem.* **1997**, *250*, 735–744.
(40) Wieprecht, T.; Beyermann, M.; Seelig, J. *Biochemistry* **1999**, *38*, 10377–10387.
(41) Surolia, A.; Swaminathan, C. P.; Ramkumar, R.; Podder, S. K. *FEBS Lett.* **1997**, *409*, 417–420.
(42) Brandts, J. F.; Lin, L.-N. *Biochemistry* **1990**, *29*, 6927–6940.
(43) Soler-Ferran, D.; Sobel, M.; Harris, R. B. *Biochemistry* **1992**, *31*, 5010–5016.