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RESEARCH ARTICLE

PROTECTIVE EFFECT OF PROLACTIN INDUCED PROTEIN ON ZINC α2-GLYCOPROTEIN AGAINST VARIOUS DENATURANTS

Md. Imtaiyaz Hassan[1,2], Aabgeena Naeem[3], Khurshid Alam Khan[2], Rizwan Hasan Khan[4] Sarman Singh[5], Tej P. Singh[1] and Savita Yadav[1,*]

[1]*Department of Biophysics, All India Institute of Medical Sciences, New Delhi 110029, India*
[2]*Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, Jamia Nagar, New Delhi 110025, India*
[3]*Department of Biochemistry, Aligarh Muslim University, Aligarh 202002, India*
[4]*Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh 202002, India*
[5]*Department of Laboratory Medicine, All India Institute of Medical Sciences, New Delhi 110029, India*

Abstract: Zinc α2-glycoprotein (ZAG) and Prolactin induced protein (PIP) are considered as important elements for fertility and biomarker for prostate and breast carcinomas. The stabilities of ZAG alone and its naturally occurring complex with PIP were compared. A significant difference in CD signal was recorded for native ZAG and ZAG-PIP complex against pH-, GdnHCl- and temperature-induced denaturation. These finding suggests that PIP plays a protective role for ZAG against several denaturants. PIP contributes to the hydrophobic as well as electrostatic interactions on ZAG for the complex formation. Moreover, the observed changes in far-UV spectra between ZAG and ZAG-PIP complex in the presence of PEG support the hydrophobic nature of the forces governing the formation of complex. This pH dependent study provides evidence that formation of the complex is a natural event required for physiological function.

Keywords: CD spectroscopy; protein-protein complex; protein stability and folding; prostate and breast carcinomas; biomarker.

Introduction

Zinc α2-glycoprotein (ZAG) and Prolactin induced protein (PIP) are present in both prostate and breast carcinomas and are designated as potential biomarker (Hassan *et al.*, 2008c; Hassan *et al.*, 2009; Katafigiotis *et al.*, 2012). ZAG is clinically a very important protein and involved in various significant biological processes (Hassan *et al.*, 2008c). Lopez-Ottin and Diamandis reviewed five proteins of common function in breast and prostate cancer and found that ZAG and PIP are also among them (Lopez-Otin and Diamandis, 1998). We have identified and purified five common proteins from the seminal fluid prostate carcinoma patients by using proteomic based approach (Hassan *et al.*, 2007). We discovered that ZAG and PIP are equally elevated in prostate carcinoma and hence considered as prognostic biomarkers for the same. We had, for the first time, identified and purified a naturally occurring complex of ZAG with PIP from human seminal fluid (Hassan *et al.*, 2008b), and found that both the proteins are held together by non-covalent interactions. Finally, we have succeeded in crystal structure determination of the complex and discovered a novel mode of association between ZAG and PIP (Hassan et al., 2008a).

The structure assembly of ZAG is also similar to immunoglobulin superfamily so that it presumably is able to bind with different proteins in seminal plasma (Hassan and Ahmad, 2011). PIP is a 14-kDa single chain secretory glycoprotein

Corresponding Author: **Savita Yadav**
E-mail: savita11@gmail.com

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present in seminal vesicles and bind with acrosomal region of spermatozoa (Chiu and Chamley, 2002; Chiu and Chamley, 2003). It strongly binds to CD-4 T cell surface receptor and Fc fragment of immunoglobulin G and mediates T-cell apoptosis (Gaubin *et al.*, 1999). The stability studies of native ZAG previously has been performed (Karpenko *et al.*, 1997). Here we present the stability of ZAG alone and in complexed form in presence of various denaturants such as high pH, temperature and salt.

Materials and Methods

Reagents and Materials: All reagents, of the highest purity commercially available, were purchased from Merck (Darmstadt, Germany), Sigma-Aldrich (St Louis, MO, USA), Fluka (St Louis, MO, USA), Applied Biosystems (Foster City, CA) and GE Healthcare (Uppsala, Sweden). Sephacryl S-200 and Resourse Q columns were from GE Healthcare. All reagents were of analytical grade. Human semen obtained from the Department of Laboratory Medicine, All India Institute of Medical Sciences, New Delhi, was initially centrifuged at 1,300 g for 15 minutes to separate spermatozoa. Phenylmethanesulfonyl-fluoride, benzamidine hydrochloride and $ZnCl_2$ were added to inhibit most of the proteases. It was further centrifuged at 13,000 g to remove debris; a clear supernatant was obtained, dialyzed against 10 mM Tris-HCl buffer, pH 8.0, which was used for purification.

Purification of ZAG-PIP complex: The native ZAG (Hassan *et al.*, 2007) and ZAG-PIP complex (Hassan *et al.*, 2008b) was purified our developed protocol. The purity of proteins was determined by SDS-PAGE, which was used for the CD measurements.

CD Measurements: The secondary and tertiary structures of native ZAG and ZAG-PIP complex were evaluated by CD spectroscopy in the far-UV (210-250 nm) and near-UV (250-320 nm) regions respectively. Far-UV spectra were measured at protein concentration of 0.2 mg/mL with a 0.1 cm cuvette, however, near-UV CD spectra was taken at 2mg/mL in 1cm quartz cuvette and was recorded at 0.1-nm wavelength intervals on a *Jasco* model 715 CD spectropolarimeter employing a scan speed of 50 nm/min and average response time of 1 second. A minimum of three consecutive scans were accumulated, and the average spectra were stored. All the CD measurements were carried with thermostatically controlled cell holder attached to a Neslab RTE 110 water bath with accuracy ± 0.1 °C. Wavelength scans at different temperature was carried out with increasing temperature from 20-85 °C at the heating rate of 1 °C /min.

Spectrophotometric Titration: Spectro photometric titration was performed in a continuous way with Schimadzu UV-60 a double beam spectrophotometer (Schimadzu Kyoto, Japan) using 1cm quartz cell and both the absorption and difference spectra of proteins were recorded. The reference cell was kept at 20 °C after 15 minutes of equilibration; the temperature of the sample cell was raised to 85 °C (1 °C/min). In addition, the cooling spectra were recorded in the same way as heating.

Results

Effect of pH

CD spectra of both native ZAG and ZAG-PIP complex at different pH were recorded in the far-UV region (210-250 nm). As can be seen from Figure 1, ZAG (curve 1 of Figure 1a) and ZAG-PIP complex (curve 1 of Figure 1b) at pH 7.5 showed a deep negative peak at 218 nm, reveals the high β-structure content. The peak started disappearing with a loss in CD signal in native ZAG, when pH was increased or decreased from 7.5. At pH 12.0 there was a complete loss in the secondary structure of native ZAG, as evident from the loss in CD signal (curve 4, Figure 1A). However, retention of structure is still clearly visible at pH 9.5, in native ZAG and ZAG-PIP complex (curve 3 of Figure 1A and B**).** On the other hand, ZAG PIP complex resist the loss in secondary structure of native ZAG either on increasing the pH to 12 (curve 4, Figure 1B) or decreasing to 2 (curve 2, Figure 1B), indicating that PIP plays a protective role for ZAG against pH denaturation. Curve 5 of Figure 1A represents native PIP protein, which is -6 [θ] degree cm^2 $dmol^{-1}x10^{-3}$ at 220 nm. On native ZAG, addition of NaCl and $CaCl_2$ does not alter the spectrum obtained at pH 12.0. However, major changes

were observed after addition of these salts in ZAG-PIP complex approaching to its original conformation i.e. pH 7.5 (data not shown). Hence this reflects the role of NaCl and $CaCl_2$ in refolding of complex.

Effect of Guanidine-Hydrochloride (GdnHCl)

ZAG is quite stable in the presence of 8.0 M urea [Karpenko *et al.*, 1997]. At low pH, urea causes a problem due to its titrable groups. Here, we used GdnHCl to predict the role of electrostatic and hydrophobic interaction involved in ZAG-PIP complex. The addition of GdnHCl up to 2.0 M led to the complete disruption of structure of native ZAG (pH 7.5) (Figure 2A). However, the structure in ZAG-PIP complex was retained up to 2.0 M GdnHCl (curve 2, Figure 2B). Further addition of GdnHCl to 6.0 M led to the loss of structure (curve 3, Figure 2B) as the peak at 218 nm completely disappeared. It is interesting to note that formation of random coil clearly visible as the peak started at below 210 nm. Further, it can be concluded that PIP plays a major role in hydrophobic as well as electrostatic interaction on ZAG after formation of complex.

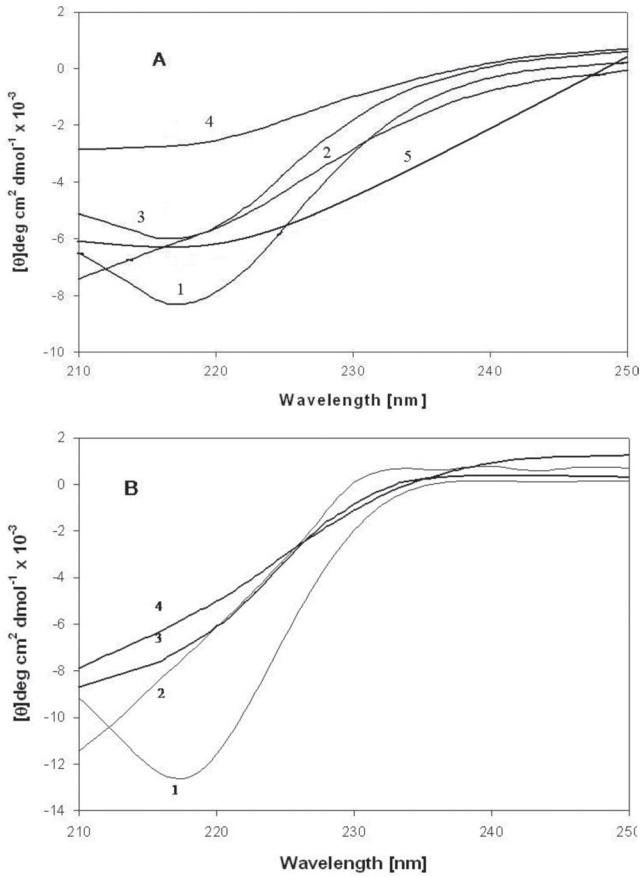

***Figure 1*: Far UV CD spectra (210-250 nm) of** (A) **Native ZAG and** (B) **ZAG-PIP complex at different pH. CD spectra of both native and complex was recorded at protein (0.2 mg/ml) in 50 mM HEPES buffer pH 7.5 (curve 1), 50 mM glycine NaOH pH 12.0 (curve 4), 50 mM glycine HCl pH 2.0 (curve 2) and 50mM sodium carbonate-bicarbonate pH 9.5 (curve 3), native purified PIP protein (curve5). The entire buffer contains 200mM NaCl and temperature was kept at 20 °C.**

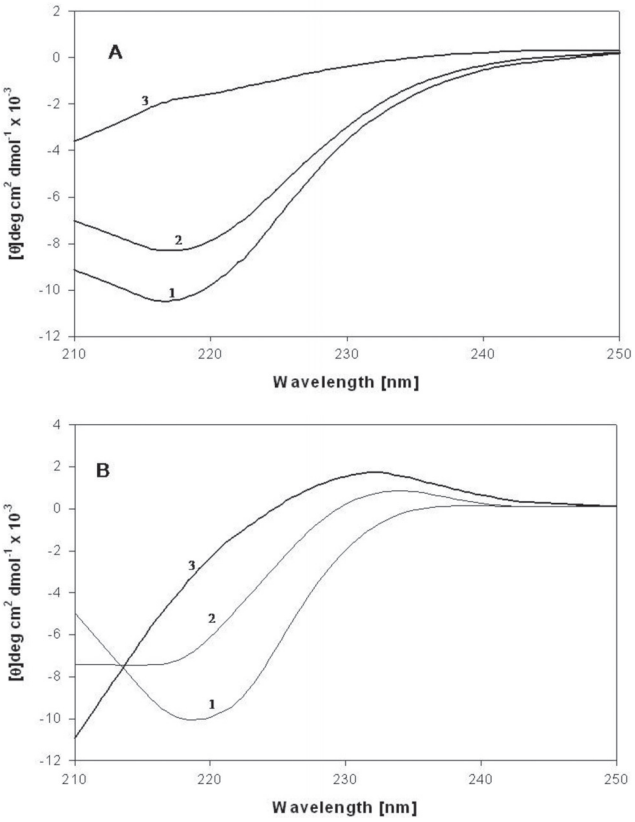

***Figure 2*: Far UV CD spectra at different concentration of GdnHCl. Spectra were recorded after incubation of protein sample in GdnHCl at 27 °C for six hour prior to record.** (A) **Native ZAG (curve 1: without GdnHCl, curve 2: 1M GdnHCl and curve 3: 2M GdnHCl).** (B) **ZAG-PIP complex (curve 1: without GdnHCl, curve 2: 1M GdnHCl and curve 3: 6M GdnHCl).**

Effect of Temperature

The changes in the secondary structure of ZAG and ZAG-PIP complex as a function of temperature were followed by CD [θ] measurements at different temperatures. The thermal transition of ZAG exhibits reversible unfolding process with an intermediate in detectable amounts. Thermal denaturation profile of ZAG and ZAG-PIP complex in the far-UV region at pH 7.5 is illustrated in Figure 3A and 3B, respectively. As evident from Figures 3A and

3B, the peaks ($[\theta]_{218}$) started disappearing gradually on increasing temperature from 20-85 °C. The recovery of ellipticity in the far-UV CD spectra indicates that the native conformation has regained upon cooling (Figure 3A and 3B, curve 3). It is interesting to note that in Figure 3B, that as the temperature increases the peak started shifting from lower to higher wavelength and at 85 °C a clear peak formed at 222 nm indicating to formation of helical structure. In the near-UV range (250-320 nm) the native ZAG showed a positive peak was observed at 265 nm, probably due to disulfide chromophores and a small positive peak, at 295 nm, is typical of aromatic chromophores (Figure 4A). This observation is in agreement with previous report of ZAG's tertiary structure. Incase of ZAG-PIP complex an unusual deep negative peak were observed at 280 nm (Figure 4B) and it is interesting that likely to native ZAG, the ZAG-PIP complex also have the same effect of temperature in tertiary structure. At 85 °C there is a complete loss of peak at 280 nm accompanied by gain in peak when cooled to 20 °C.As the peak at 218 nm formed in far-UV range we have heated the proteins from 20 to 85 °C, which should be used to describe the temperature dependence of the changes in ellipticity signal (data not shown). But in the case of ZAG-PIP complex an unusual pattern were appeared on increasing temperature from 20 to 85 °C. On increasing temperature up to 40 °C is stabilizing the structure as the mean ellipticity value is increasing towards negative side (data not shown). On further increase in temperature causes complete denaturation. The presence of PIP in the ZAG shows stability up to 65 °C, beyond that there is a loss in structure. Interestingly, our thermal denaturation profile of native ZAG is consistent with the reported value

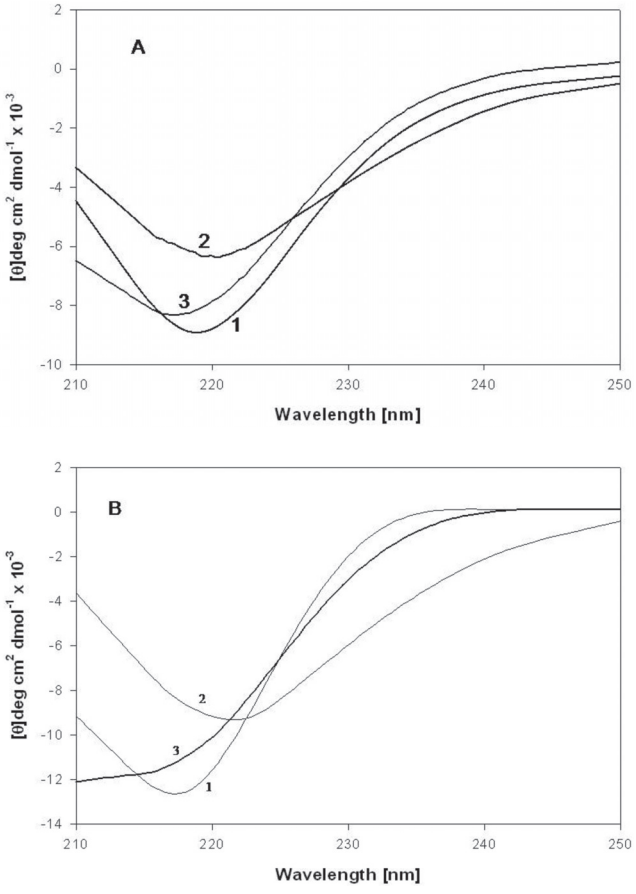

Figure 3: **Spectra of native ZAG and ZAGPIP complex was recorded at different temperatures** (A) **Native ZAG and** (B) **Spectrum of ZAG-PIP complex at 210 nm to 250 nm in far UV range. curve 1: at 20 °C, curve 2: 85 °C and curve 3: after cooling from 85 °C to 20 °C.**

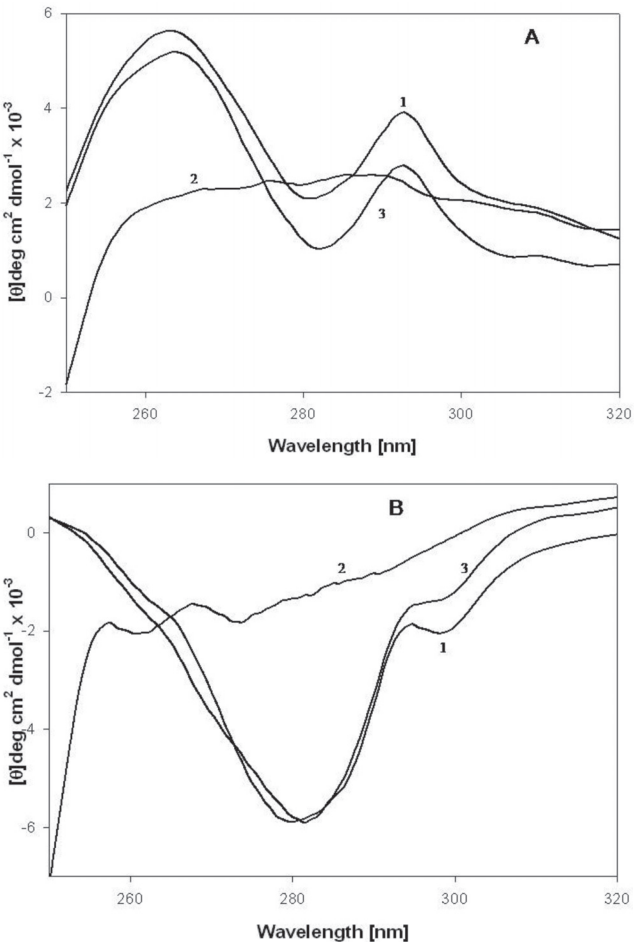

Figure 4: **Thermal denaturation curves of** (A) **Native ZAG and** (B) **ZAG-PIP complex was recorded between and 250-320 nm in near UV range. Curve 1: at 20 °C, curve 2: 85 °C and curve 3: after cooling from 85 °C to 20 °C.**

for serum ZAG (Karpenko *et al.*, 1997). However, such parameter is considerably different in the case of ZAG-PIP complex.

Effect of Polyethylene glycol 200 and 400 (PEGs)

Changes in Far-UV CD spectra in 210-250 nm of ZAG and ZAG-PIP complex in combination with different concentration of PEG400 were observed (Figure 5A and 5B). The absorption of PEG alone was taken into account and this study reports subtracted spectrum. The native ZAG exhibited a trough at 218 nm, which is a characteristics feature of β-conformation in the proteins. At 1% (v/v), PEG 400, no change was observed in the native ZAG and with successive increase in PEG concentration up to 2% (v/v) there was a loss of trough at 218 nm with decrease in negative CD signal. At 3% (v/v) PEG there was a complete loss of secondary structure of native ZAG (Figure 5A). On the other hand, CD spectra showed remarkable changes in ZAG-PIP complex when PEG was added. There was a complete loss of structure at 2% (w/v) of PEG only. This indicates that PEG destroys all the hydrophobic forces between ZAG and PIP, which requires them for complex formation. Disruption of structure with the use of PEG indicates that the main forces between ZAG and PIP are hydrophobic in nature. It can also be elucidated that PEG doesn't alter the complete structure in both the cases only the slight disappearance has been observed at 218 nm.

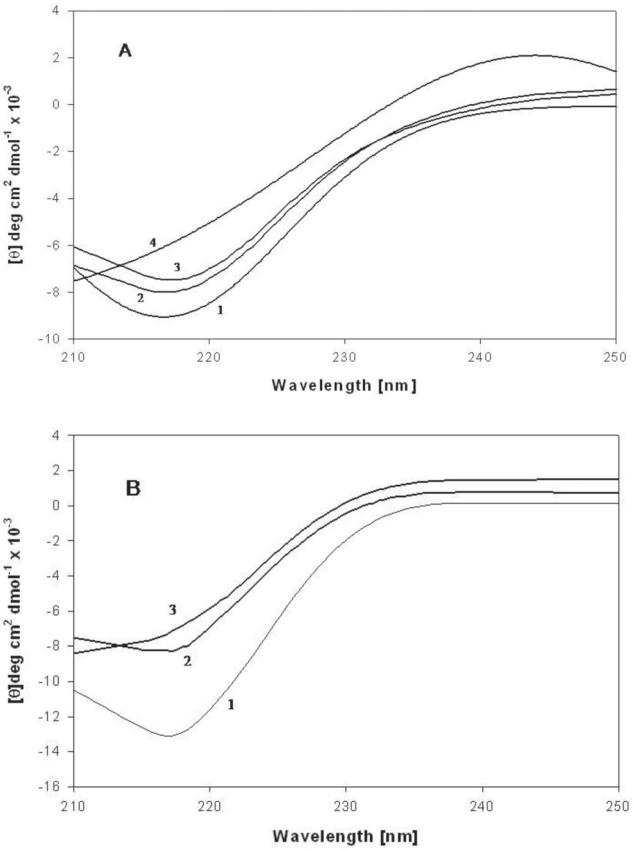

***Figure 5*: PEG dependent denaturation of** (A) **Native ZAG and** (B) **ZAG–PIP complex. The concentration of PEG 400 increased linearly from 1% to 3% (curve 2-4) upon successive addition to the sample with 15 min incubation at 20 °C. The absorption observed by PEG has been subtracted.**

Discussion

The far-UV CD spectra of ZAG showed a clear peak indicating the predominant presence of β-pleated structure, as also evident from the crystal structure of ZAG, which contains four helices while the rest are β-strands in the form of sheets (Sanchez *et al.*, 1999). The spectra of ZAG-PIP complex have the same feature; instead they have more ellipticity in the region of 218 nm without remarkable changes in 222 nm indicating that PIP is also a beta rich protein. That is clearly observed in the crystal structure of ZAG-PIP complex where PIP is comprised of seven β-strands in the form of β-pleated sheet (Hassan *et al.*, 2008a). Increasing the pH to 9.5 or decreasing to 2 have almost the same structural changes both in native ZAG or ZAG-PIP complex. At pH 12, most of the charged residues become negatively charged and the charge-charge repulsion is accompanied by loss in structure. We have added NaCl in the denatured protein to minimize the charge-charge repulsion. It is interesting to note that native ZAG doesn't regain their structure even at 200 mM NaCl in contrast to the ZAG-PIP complex that retains half of the structure at 200 mM NaCl. It reveals that in addition to hydrophobic interactions, ZAG-PIP is also held together by hydrogen bonds and this is in agreement with our previously determined crystal structure. On the other hand PIP also plays a protective role against GdnHCl and finally participates in structure stabilization.

The CD spectrum of ZAG-PIP complex showed maximum changes in the region of 210-220 nm. At 40 °C, in the ZAG-PIP complex, there was loss of typical β-sheet structure with the appearance of two minor peaks at 218 and 222

nm as observed in Figure 3B. At 85 °C, there was a complete loss of secondary structure as indicated by an increase in negative CD signal, which may be due to aggregation of protein not visible to the naked eye. However, on decreasing the temperature to 20 °C, ZAG recovered its native spectral features with an enhanced negative CD peak at 218nm. Interestingly, when ZAG-PIP complex was cooled, it retained its tertiary structure. The data indicates that native ZAG as well as ZAG-PIP complex is susceptible to thermal denaturation but in a reversible manner. ZAG has relatively high content of aromatic amino acid residues (Trp-2.9% and Tyr-6.5%); its resistance to the heat-induced changes in the region of 230-240 nm, indicates that sharp wavelength aromatic transition can influence the CD spectrum. The near UV CD spectra of native ZAG and ZAG-PIP are entirely different. While the molecular details of such differences cannot be ascertained, we can predict that after forming the complex the tertiary structural organization undergoes changes that may lead to the change in near UV CD spectra. Interestingly, both native ZAG and ZAG-PIP complex have shown reversible changes in secondary as well as tertiary structure. PEG causes perturbation in hydrophobic interactions, which is the main driving force to maintain structure and assembly in proteins. In most of the cases native ZAG was more susceptible to denaturation as compared to ZAG-PIP complex. However, the ZAG-PIP complex is denatured at only 2% of PEG suggesting that PEG may cause change in surface morphology.

ZAG is quite stable in absence of peptide and β_2-microglobulin (β_2M), unlikely for Class I MHC molecule which requires bound peptide for structural stability (Fahnestock *et al.*, 1992; Sanchez *et al.*, 1997). ZAG is stably folded in the absence of bound peptide and β_2M (Sanchez *et al.*, 1997). We compared the thermal denaturation profile of native ZAG with denaturation profiles of ZAG-PIP complex. The melting curve of native ZAG is similar to the peptide bound MHC-I molecules with a T_m value corresponding to 65 °C (Sanchez *et al.*, 1997). However, the ZAG-PIP complex has T_m value much higher than that and attains extra stability due to binding of PIP. The PIP is present in the cleft formed by α1-α2 and α3 domains where PIP strongly binds and forms several non-covalent interactions with ZAG (Hassan and Ahmad, 2011; Hassan *et al.*, 2008a). PIP is known to bind with the CD-4 T cell receptor which involves antigen presentation and processing. The structural stability and similararity to β_2M like light chain counterpart (PIP) of ZAG indicates that its function has diverged towards peptide presentation and T cell interaction, likely the functions attributed to class I MHC molecules. Our results are consistent and showed that binding of PIP to ZAG further enhanced the stability of ZAG.

The complex formed between ZAG and PIP is quite stable because β-strand of PIP antiparallely fitted with β-strand of ZAG which is similar to PapD-PapK complex (Sauer *et al.*, 1999). Due to experimental barrier and unavailability of purified PIP; we have not used PIP alone as a blank. However, the intensity of changes in curves under harsh conditions is clearly indicative of the protective role of PIP for ZAG against denaturation. All these findings suggest that such novel mode of association between ZAG and PIP presumably occurs for stabilizing these proteins in the vaginal tract where pH is very low.

Conclusions

This manuscript describes the conformational stability studies on ZAG alone and in complex with PIP. The CD based stability studies reveal that a stable complex has been formed between ZAG and PIP. ZAG and PIP are held together by strong non-covalent interactions and PIP provides additional stability to ZAG by holding its α1-α2 domain platform with α3 domain. From the structure and biochemical analysis it is evident that the complex of ZAG and PIP is reasonably stable to temperature and other denaturants commensurate with its localization and physiological function. Further work is needed to compare the stability of ZAG and ZAG-PIP by using different probes.

Acknowledgements

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Abbreviations

CD, Circular dichroism; DLS, Dynamic light scattering; Gdn HCl, Guanidine Hydrochloride; PEG, Polyethylene glycol; PIP, Prolactin induced protein; ZAG, Zinc α2-glycoprotein; ZAG-PIP, Zinc α2-glycoprotein and prolactin induced protein complex.

References

Chiu, W. W., and Chamley, L. W. (2002). Antibody-binding proteins in human seminal plasma. Am J Reprod Immunol *48*, 269-274.

Chiu, W. W., and Chamley, L. W. (2003). Human seminal plasma antibody-binding proteins. Am J Reprod Immunol *50*, 196-201.

Fahnestock, M. L., Tamir, I., Narhi, L., and Bjorkman, P. J. (1992). Thermal stability comparison of purified empty and peptide-filled forms of a class I MHC molecule. Science *258*, 1658-1662.

Gaubin, M., Autiero, M., Basmaciogullari, S., Metivier, D., Mis hal, Z., Culerrier, R., Oudin, A., Guardiola, J., and Piatier-Tonneau, D. (1999). Potent inhibition of CD4/TCR-mediated T cell apoptosis by a CD4-binding glycoprotein secreted from breast tumor and seminal vesicle cells. J Immunol *162*, 2631-2638.

Hassan, I., and Ahmad, F. (2011). Structural diversity of class I MHC-like molecules and its implications in binding specificities. Adv Protein Chem Struct Biol *83*, 223-270.

Hassan, M. I., Bilgrami, S., Kumar, V., Singh, N., Yadav, S., Kaur, P., and Singh, T. P. (2008a). Crystal structure of the novel complex formed between zinc alpha2-glycoprotein (ZAG) and prolactin-inducible protein (PIP) from human seminal plasma. J Mol Biol *384*, 663-672.

Hassan, M. I., Kumar, V., Kashav, T., Alam, N., Singh, T. P., and Yadav, S. (2007). Proteomic approach for purification of seminal plasma proteins involved in tumor proliferation. J Sep Sci *30*, 1979-1988.

Hassan, M. I., Kumar, V., Singh, T. P., and Yadav, S. (2008b). Purification and characterization of zinc alpha2-glycoprotein-prolactin inducible protein complex from human seminal plasma. J Sep Sci *31*, 2318-2324.

Hassan, M. I., Waheed, A., Yadav, S., Singh, T. P., and Ahmad, F. (2008c). Zinc alpha 2-glycoprotein: a multidisciplinary protein. Mol Cancer Res *6*, 892-906.

Hassan, M. I., Waheed, A., Yadav, S., Singh, T. P., and Ahmad, F. (2009). Prolactin inducible protein in cancer, fertility and immunoregulation: structure, function and its clinical implications. Cell Mol Life Sci *66*, 447-459.

Karpenko, V., Kaupova, M., and Kodicek, M. (1997). The conformation and stability of human Zn-alpha 2-glycoprotein in aqueous and methanolic solutions. Biophys Chem *69*, 209-217.

Katafigiotis, I., Tyritzis, S. I., Stravodimos, K. G., Alamanis, C., Pavlakis, K., Vlahou, A., Makridakis, M., Katafigioti, A., Garbis, S. D., and Constantinides, C. A. (2012). Zinc alpha2-glycoprotein as a potential novel urine biomarker for the early diagnosis of prostate cancer. BJU Int. in Press.

Lopez-Otin, C., and Diamandis, E. P. (1998). Breast and prostate cancer: an analysis of common epidemiological, genetic, and biochemical features. Endocr Rev *19*, 365-396.

Sanchez, L. M., Chirino, A. J., and Bjorkman, P. (1999). Crystal structure of human ZAG, a fat-depleting factor related to MHC molecules. Science *283*, 1914-1919.

Sanchez, L. M., Lopez-Otin, C., and Bjorkman, P. J. (1997). Biochemical characterization and crystalization of human Zn-alpha2-glycoprotein, a soluble class I major histocompatibility complex homolog. Proc Natl Acad Sci U S A *94*, 4626-4630.

Sauer, F. G., Futterer, K., Pinkner, J. S., Dodson, K. W., Hultgren, S. J., and Waksman, G. (1999). Structural basis of chaperone function and pilus biogenesis. Science *285*, 1058-1061.