Discussion
5. DISCUSSION

I. Purification and characterization of Human Placental Cystatin

Natural inhibitors of cysteine proteinases are called as cystatins. They constitute a powerful regulatory system for endogenous cysteine proteinases, which may otherwise lead to uncontrolled proteolysis and tissue damage. Cystatin are the Superfamily of evolutionary, structurally and functionally related proteins involved in the inhibition of papain and related cysteine proteinases, cathepsins B, H, L, ficin and bromelain (Barrett 1981; Abrahamson 1994). All cystatins form a single superfamily within which all the members can be shown to have a statistically significant relation with each other or with papain but they do not resemble members of other superfamilies (Dayhoff et al., 1979 a,b). Members of the superfamily may be divided in to three families namely stefins, cystatins and kininogens on the basis of sub-cellular localization, disulphide bonds, number of amino acid residues and sequence homology, (Barrett et al., 1986a).

The unique properties and wide range of physiological function of thiol proteinases are remarkable and demand attention. A proteinase inhibitor is of physiological importance because inhibition is achieved at physiological concentration of the inhibitor in a sufficiently short time with negligible dissociation of the complex (Beith 1980). They are associated with several pathological conditions such as rheumatoid arthritis (Trabandt et al., 1991), renal failure (Kabanda et al., 1995), septic shock (Assfal-Machleidt et al., 1988), osteoporosis (Delaisse et al., 1991), metastasizing cancer (Koppel et al., 1994), purulent bronchiectasis (Buttle et al., 1990) and peridontitis (Cox and Eley, 1989) resulting due to imbalance of endogenous cysteine proteinase inhibitors (CPI’s) and the proteinases. Extensive work has been carried out for the purification and characterization of these inhibitors using various isolation procedures. Purification of CPI’s have been reported from various mammalian tissues like bovine muscle (Zabari et al., 1993), chicken egg white (Barrett 1986), rat submandibular cystatin
(Bedi 1989) and rabbit skeletal muscle (Matsuishi and Okitani, 2003), epidermis (Ohtani et al., 1982a), various squamous epithelia (Hopsu-Havu et al., 1983; Ohtani et al., 1982b), liver (Green et al., 1984) and spleen (Jarvinen and Rinne, 1982).

In the present study thiol proteinase inhibitor has been purified from human placenta an organ of special importance in human system. In normal pregnancy and various pregnancy associated disorders, placenta plays highly significant role (Lin et al., 1976). Lysosomes present in the cells of placenta take part in the intracellular degradation of proteins (Bohley et al., 1978). The activity of lysosomal enzymes (cathepsins B, H, L and C) is controlled by cystatins present in the placenta. Since lacuna exists in the isolation and physicochemical characterization of cystatin from this tissue it was envisaged that a thorough and systematic study will be useful in understanding in depth about the cystatins of human placenta and to compare its properties with other known cystatins.

The cystatin was purified from human placenta using a simple procedure which involved ammonium sulphate precipitation and gel filtration chromatography (Fig 4.0) (Rashid et al., 2004). The procedure is rapid with a 14.8% yield and 8913 fold enrichment in specific activity of the protein (Table 4.0). The purification scheme gave a better yield as compared to the method of Warwas and Sawicki (1989) which is a multistep procedure with 13% yield and lower specific activity. Purification of CPI from other sources has been reported using affinity chromatography, chromatofocussing, gel filtration and ion exchange chromatography (Anastasi et al., 1983; Evans and Barrett, 1987; Cimermann et al., 1996).

The purified inhibitor was found to be homogenous on the basis of charge and molecular weight as shown by native-PAGE (Fig 4.1) and SDS-PAGE in non-reducing and reducing conditions (Fig 4.2). The results suggest that the purified protein is a single polypeptide. The molecular weight obtained by passing marker proteins through Sephadex G_{50,80} column was calculated to be 12500 (Fig 4.3). It was further confirmed by SDS-PAGE and the value obtained was 12445 (Fig 4.4). Cystatins type 1 and type
have been classified on the basis of molecular weight and presence of disulphide bonds (Abrahamson 1994). Cystatins from tissues are usually small inhibitors having molecular mass in the range of 11 to 25 KDa (Barrett 1984). Molecular weights of 11400 and 12000 have been reported for cysteine proteinases inhibitors isolated from Human spleen (Jarvinen and Rinne, 1982), 14000 and 14300 for cystatins from bovine muscle (Zabari et al., 1993), 14200 from human liver (Green et al., 1984). For type 1 cystatins (Stefins A and B) molecular weights of 11100 and 12000 have been reported (Turk et al., 1995). Hence, the molecular weight of cystatin obtained in the present study suggests it to be a type 1 cystatin.

Hydrodynamic parameters for the HPC as determined from gel filtration behaviour suggested a stokes radius of 14.5 Å (Fig 4.5) and diffusion coefficient of \( 1.53 \times 10^{-14} \text{ cm}^2/\text{sec} \). Diffusion coefficient depends on the size and shape of the molecule. The stokes radius of cytochrome C is 16.4 Å, it is a compact and globular protein. Thus as indicated by the hydrodynamic properties human placental cystatin has a compact globular structure.

Generally, cystatin type 1 and type 2 which are isolated from tissues lack carbohydrate content (Barrett et al., 1984) where as presence of carbohydrate content is a distinguishing property of type 3 cystatins, the kininogens (Salvesen et al., 1986). However, presence of carbohydrate has been reported in some type 2 cystatins for example, rat cystatin C isolated from urine is slightly glycosylated (Esnard et al., 1988). Ni et al (1997) found carbohydrate attachments in cystatin E. Glycosylation has also been demonstrated in cystatin F and cystatin M which are all type 2 cystatins (Ni et al., 1998; Sotiropoulou et al., 1997). Human Placental cystatin is devoid of any carbohydrate content and disulphide bonds are also absent which is again a characteristic similar to type 1 cystatins (Barrett et al., 1984).

Effect of pH on human placental cystatin activity showed that the inhibitor has stable activity in the pH range 3.0-9.0 (Fig 4.6). Further, this inhibitor was found to be stable in the temperature range 40°C to 100°C (Fig 4.7). The inhibitor was stable for 120 minutes at 90°C (Fig 4.8). These
properties are in accordance with other cystatins, from bovine muscle (Beige et al., 1985) and Chicken cystatin (Saxena and Tayyab 1997). Zerovnik et al (1997) have shown high thermal stability of chicken cystatin and stefin A and B. Barrett et al (1986b) reported similar stability for LMW-CPI up to 100°C for 5 min.

The placental cystatin showed moderate immune response and gave a low titre of antibodies in rabbits owing to its low molecular weight. The titre of antibodies determined by direct binding ELISA in rabbit serum was 2238.73 (Fig 4.9). The low antibody titre suggests that the inhibitor used as antigen here is of low molecular weight range. The antibodies raised against purified inhibitor gave a reaction of identity with the inhibitor as indicated by a single precipitin line on immunodiffusion indicating that HPC has immunogenic purity and homogeneity (Fig 5.0). The antisera exhibited no immunogenic identity with sheep plasma HMW kininogen and phytocystatin purified from Phaseolus mungo (Urd-a commonly used Indian legume) (Fig 5.1). This indicates that the human placental cystatin has no immunogenic identity or similarity in structure and composition to high molecular weight mammalian kininogens (type 3 cystatins) and to phytocystatins.

Stoichiometry of the binding of human placental cystatin with papain, ficin and bromelain was determined by the titration of the proteases with the inhibitor. A stoichiometric ratio of 1:1 was obtained for HPC with papain, ficin and bromelain. This suggests that human placental cystatin is a tight binding inhibitor of these proteases. Anastasi et al (1983) and Nicklin and Barrett (1984) reported equimolar complexes of cystatin with cysteine proteinase. Abrahamson et al (1987) also reported the rapid formation of 1:1 complex between cystatin C and papain. The human placental cystatin (HPC) strongly inhibits thiol proteinases- papain, ficin and bromelain but does not inhibit serine proteinases like trypsin and chymotrypsin (Fig 5.2). This has also been reported for many other cystatins along with the thiol proteinase inhibitor isoforms purified from human spleen (Jarvinen and Rinne, 1982).
The inhibitory activity of HPC towards above mentioned thiol proteinases and serine proteinases were examined using casein as substrate. Accurate Ki values were determined by working at lower enzyme concentrations and using equations derived by Krupka and Laidler (1959) and Henderson (1972). Ki values were calculated from the slope of curve obtained for the inhibition of caesinolytic activity of papain, ficin and bromelain (Table 4.1). HPC is a potent inhibitor of thiol proteinases as is evident by their Ki values. It was readily apparent that the degree of inhibition varied with the absolute concentration of the inhibitor as is expected for tight binding reversible inhibition. The data shows that HPC inhibited papain, ficin and bromelain with Ki values of $5.5\times10^{-8}$ M, $8.4\times10^{-8}$ M and $9.5\times10^{-8}$ M respectively, under conditions of routine assay system (Fig 5.3, 5.4 and 5.5). The Ki value is lowest for papain hence it has the highest affinity for the inhibitor. These values are in good comparison with other thiol proteinase inhibitors, for example inhibition constant (Ki) for chicken cystatin and cystatin C has been reported to be $5\times10^{-12}$ M with papain, Ki values of nanomolar range have been reported for cathepsin B, H and L with cystatin A (Barrett et al., 1984), cystatin C (Machleidt et al., 1986), chicken cystatin (Salvesen et al., 1986) and cystatin D (Balbin et al., 1994). The increasing value of Ki with an increase in substrate concentration suggests the inhibition to be competitive. This finding is supported by the apparent results of Nicklin and Barrett (1984) for the inhibition of human cathepsin B by chicken cystatin. They obtained Ki (app) values of 1.85 and 3.68 nM with the substrate concentration of 0.05 and 0.39 mM, respectively.

IC$_{50}$ value is the concentration of the inhibitor at which 50% of the enzyme activity is inhibited. IC$_{50}$ values of the human placental cystatin for the three proteinases papain, ficin and bromelain were 0.051 μM, 0.091μM and 0.131μM respectively (Table 4.1). Lesser IC$_{50}$ value suggests a greater affinity of the inhibitor towards the enzyme. Thus, these values indicate that the affinity of HPC for these proteinases are in order of papain> ficin> bromelain. Katunuma and Kominami (1985) have found the IC$_{50}$ value of
0.16 µg of the inhibitor isolated from rat liver for papain and 0.46 µg for ficin and IC<sub>50</sub> value of 4.2 µg for cathepsin B and 0.14 µg for cathepsin H.

The kinetics of association was determined assuming that the enzymes and the inhibitor react in such a way that dissociation rate is low enough to neglect the reverse reaction during the initial part of the reaction. The slope of the straight line obtained by plotting 1/ [E] versus time for papain, ficin and bromelain gave a k<sub>+1</sub> values of 3.4x10<sup>4</sup>, 2.9x10<sup>3</sup> and 8.6x10<sup>2</sup> (M<sup>-1</sup>S<sup>-1</sup>) respectively (Fig 5.6, 5.7 and 5.8). Again the affinity was in the order of papain > ficin > bromelain. The dissociation constant values (k<sub>-1</sub>) for the enzyme-inhibitor complex was determined by displacement procedure, in which the inhibitor released from the complex was trapped by excess substrate (casein) with the increase in time. The amount of enzyme released from the complex was monitored by continuous measurement of enzyme activity. The respective k<sub>-1</sub> values of HPC obtained for papain, ficin and bromelain were 2.3x10<sup>-5</sup>, 2.6x10<sup>-5</sup> and 2.1x10<sup>-4</sup> (S<sup>-1</sup>) respectively (Fig 5.9, 6.0 and 6.1). The values of k<sub>-1</sub> are consistent with k<sub>-1</sub> values obtained for chicken cystatin, 5x10<sup>-5</sup> S<sup>-1</sup> with papain and 2.3x10<sup>-3</sup> S<sup>-1</sup> with cathepsin B (Nicklin and Barrett, 1984). The calculated half life values of enzyme-HPC complex using these k<sub>-1</sub> values for papain, ficin and bromelain were 3.01x10<sup>-4</sup> S, 2.6x10<sup>-4</sup> S and 3.3x10<sup>-4</sup> S respectively (Table 4.1). The above data gives comprehensive information about the kinetics of inhibition of purified cystatin with papain, ficin and bromelain and the overall comparison showed that HPC inhibits papain more effectively compared to other two proteinases.

The sequence of N-terminal 30 amino acid residues gave some interesting results. As reported for other human cystatins, the human placental cystatin has Gly residue at position 11 rather than the conserved position 9 in various species (Brzin et al., 1984) however, maximum sequence homology was observed with chicken cystatin (Schwabe et al., 1984). Sequence homology of HPC has also been observed with Bovine cystatin (Zabari et al., 1993), human-salivary cystatin SN, cystatin S7, cystatin C (Turk et al., 1995), rat cystatin (Bedi 1989), human-stefin A

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(Machleidt et al., 1983) stefin B (Ritonja et al., 1985) and Rat liver TPI (thiol proteinases inhibitor) (Takio et al., 1983), the details are given in Table 4.2. Using the sequence of these N-terminal amino acid residues a hydropathy plot was made using the respective hydropathy index of each individual amino acid (Fig 6.2). It was observed that among the 30 residues sequenced the stretch of 4-8, 8-12 and 20-24 residues have maximum average hydropathy index indicating that these residues are present inside the hydrophobic core of the protein.

The interaction between papain and human placental cystatin was studied using various spectroscopic techniques. UV absorption difference spectra between the HPC-papain complex showed a pattern of changes in ultraviolet absorption in the aromatic wavelength region (Fig 6.3). The spectrum shows maxima at 295 nm and 255 nm and minima at 280 nm. The peak at 295 nm suggests that aromatic residues are involved in binding with papain where as the peak at 280 nm is indicative of changes around tyrosine residues (Donovan 1969; 1973a; 1973b). The peak around 255nm may be partly due to phenyalanine or cysteine residues and may also contain contribution from aromatic residues (Donovan 1973a). Our results are comparable to positive absorption difference spectrum throughout the aromatic wavelength region reported earlier for rat cystatin-papain interaction (Takeda et al., 1986). These results suggest that the environment of several aromatic amino acids has been perturbed by this interaction.

Fluorescence studies showed a decrease in wavelength of the maximum fluorescence emission spectrum of cystatin on binding with papain (Fig 6.4). The maxima of cystatin shifted from 345 nm to 340 nm for cystatin papain complex. This blue shift was accompanied by a small decrease of maximum emission intensity. The fluorescence changes induced by the binding further shows that the environment of one or more aromatic side chains is perturbed (Friefelder 1982). These changes may have arisen due to altered conformation of either or both the proteins induced by their binding to form the complex (Rashid et al., 2004).

CD spectra in the far UV region depict the contribution of the
secondary structure of the protein (Jiregensons 1970). The α-helical structure of the protein in the far UV region is characterized by negative peaks at 208-210 nm and 222 nm and a positive peak between 190-192 nm (Jiregensons 1970, Chen et al., 1972). HPC has α-helical content of 21.08% and also shows presence of significant amount of β structure (Fig 6.5). The α-helical content was calculated from the ellipticity values at 222 nm using equation given by Chen et al (1972). This type of structure has also been reported for chicken cystatin which has α-helical content of about 20% with substantial amount of β-structure (Schwabe et al., 1984). Structurally, the resemblance between chicken cystatin which is a type 2 cystatin and steffins A and B which are type 1 cystatins has been reported earlier (Martin et al., 1995; Stubbs et al., 1990). Stefin A and stefin B have an α-helical content of 22% and 20% respectively, however, chicken cystatin has disulphide bonds which are absent in case of HPC as well as in the steffins A and B, in addition there are differences in amino acid sequence (Staniforth et al., 2001).

The placental cystatin loses its native structure on formation of complex with papain (Fig 6.5). The complex show’s positive ellipticity in the range of 215-240 nm’s and small shoulders appear in 217-230 nm region which is a feature of random coil structure (Chen et al., 1972). Far UV-CD spectroscopic changes shown here on binding of human placental cystatin to papain are appreciably different from those reported earlier for the interaction of chicken cystatin with papain (Lindahl et al., 1988). The results reported by Lindahl et al (1988) showed small changes in far UV-CD spectrum of the complex of chicken cystatin and papain. Complex spectroscopic changes in the far UV-region accompanying the binding of human placental cystatin to papain indicates that the environment of several aromatic amino acids in one or both the proteins is perturbed by the interaction resulting in conformational changes in the constituent proteins.

The results indicate that the UV absorption, fluorescence emission and far UV-CD changes are more due to conformational changes in proteins rather than any local interaction affecting chromophoric groups of the two
constituent proteins of the complex. The positive ellipticity observed for PI (protein-inhibitor) complex in far-UV CD region further confirms that cystatin and papain both lose their native structures on forming complex. The conformation of this complex resembles neither of the constituent proteins, rather indicates attainment of random coil structure due to this interaction. The kinetics studies also suggest formation of tight complex on interaction of inhibitor with papain (Rashid et al., 2004).

Thus, owing to absence of any carbohydrate content and disulphide bonds and all other results taken together, HPC can be placed in type 1 cystatin family with resemblance to chicken cystatin which is a type 2 cystatin showing no structural similarity with type 3 cystatins (kininogens).

II A. GdnHCl and Urea induced Unfolding of HPC

GdnHCl and Urea are the most common chemical denaturants, used for probing the protein conformation, stability and unfolding studies. These denaturants show different behaviour towards different proteins, for example, prostaglandin D-Synthase in presence of urea and GdnHCl show an increase in enzyme activity at lower concentrations which is a deviation from classical behavior of denaturants (Inui et al., 2003). In case of protein matrilysin (recombinant human matrix metalloproteinase 7) half of the fluorescence change was observed at 2.2-2.7 M GdnHCl, where as no change was observed even with 8M urea and half inactivation of protein was induced at 0.8 M GdnHCl and at 2 M urea (Inouye et al., 2000). It is generally recognized that protein denaturation is a highly cooperative process, which for small globular proteins may be approximated by a two-state model and no significant intermediates are present during the transition N —> D (Aune and Tanford, 1969). However, recently reported results show that some intermediates exist during the unfolding of proteins. The intermediates between native and unfolded states have been referred to as molten globules in some cases (Ptitsyn, 1995). Such intermediates have been found for many proteins in mild denaturing conditions.
The protein molecule in the molten globule state (MG) is almost as compact as in the native state (Dolgikh et al., 1981; Gast et al., 1986; Uversky et al., 1993) and has a loosely packed non-polar core. It is usually characterized by intense far-UV CD spectra, which suggests existence of pronounced secondary structure (Kuwajima, 1989). In case of α-lactalbumin the amplitude of CD spectrum increases in the molten globule state compared to that of native conformation (Vassilenko and Uversky, 2002). For several proteins (Shortle, 1996; Mork et al., 1999) when NMR data has been combined with hydrodynamic and small angle scattering data the picture emerges that the protein chains are relatively compact under mild denaturating conditions, forming intermediates (Shortle, 1996; Mork et al., 1999). As conditions are made less favorable for structure formation generally by adding chemical denaturants like GdnHCl and urea, the denatured state gradually loses its residual structure and increases in size (Dill and Shortle, 1991). At the highest concentration of denaturant, the conformation is expected to converge towards that of a random coil (Shortle and Ackerman, 2001).

Differences in the behaviour of HPC have been observed from classical behaviour towards GdnHCl and urea. Activity measurements show that at 1.5M GdnHCl concentration the enzyme is already inactivated by 45% suggesting a change in conformation of protein compared to fully active native form (Fig 6.6). Two different effects are displayed by GdnHCl on the unfolding pattern of HPC. At low concentrations (0.5 to 1.5M) the protein shows increased ellipticity as compared to the native state and quenching of fluorescence in this concentration region compared to that of zero denaturant concentration. The decrease in fluorescence intensity observed up to 1.5M may be caused by tertiary structural rearrangement involving aromatic residues or because of the increased mobility of the local environment of the aromatic residues. It could be due to aggregation which quite often occurs in non native states, even a combination of these factors may also be responsible for this decrease. Normally exposed aromatic residues in the unfolded protein show emission maxima between 348-356nm.
Treatment of HPC with higher concentration of GdnHCl and urea has resulted in exposure of buried residues present in the native protein to the solvent as $\lambda_{\text{max}}$ in case of GdnHCl red shifts to 350nm and in case of urea to 348nm, suggesting induction of unfolding of HPC by these denaturants (Fig 6.7 A and B).

GdnHCl starts behaving as a classical denaturant at higher concentrations where it leads to extreme unfolding of the enzyme. However, in case of urea the transition curves of unfolding are sigmoidal in shape where the protein inactivates and loses its conformation as the concentration of denaturant increases giving a mid-point of transition at 3M, where as in case of GdnHCl there is tendency of forming intermediates at low concentration regions with mid-point of transition in the range 1.5 → 2M (Fig 6.7 C and 6.8 C). The increased ellipticity at lower concentrations of GdnHCl may be due to transformation of native form to some intermediate state which is different from both native and completely denatured states (Fig 6.8 A) where as in case of urea denaturation there is gradual decrease in ellipticity of protein with increase in urea concentration without formation of any intermediate states (Fig 6.8 B). This formation of intermediates can have several explanations. It may be due to micro-environmental changes in the aromatic region of the protein, small local rearrangements of the native state (Ferreon and Bolen, 2004) or the stabilizing effects of GdnHCl at low concentrations (Mayr and Schmid, 1993; Smith and Schlotz, 1996). The data obtained for HPC denaturation is supported by several other reports on GdnHCl and urea providing different estimates for the conformational stability of proteins (Pace, 1975; Yao and Bolen, 1995; Park et al., 2003; Deshpande et al., 2003; Wang et al., 2000; Inui et al., 2003; Inouye et al., 2000).

The difference in behavior of GdnHCl and urea towards HPC denaturation may also be explained on the following basis. Although, urea and GdnHCl have similar modes of action, urea has only chaotropic effects where as GdnHCl is a monovalent salt having both ionic and chaotropic effects. Guanidine is an electrolyte with pKa of 11, which means that at pH
values below this it will be present in a fully protonated form as Gdn\(^+\). The presence of Gdn\(^+\) and Cl\(^-\) influence the stability properties of proteins and enzymes. The effect of GdnHCl and NaCl has been reported on RNase T1 (Mayr and Schmid, 1993) where compaction of native enzyme is interpreted in terms of stabilization by cation (Gdn\(^+\) and Na\(^+\)) binding to the negatively charged moieties of protein. There is stabilization of enzyme by affinity binding of these cations at one or more sites, this effect has also been demonstrated for glucose oxidase (Ahmad et al, 2001).

In the present case also, HPC may be stabilized by low concentration of cation binding to the negatively charged sites of the inhibitor. Hence at low concentration of GdnHCl, stabilization by Gdn\(^+\) cation binding to negatively charged sites in protein dominates and at higher concentrations it acts as a classical denaturant leading to unfolding of protein chain. As protein contain ion-binding sites of varying affinity and specificity degree of stabilization of proteins by denaturants could vary from protein to protein. The unfolding of human placental cystatin suggests different unfolding pathways and mechanisms for the two denaturants. The urea unfolding corresponds to a two-state mechanism where N\(\rightarrow\)D transition takes place in a single step and no intermediate states are observed. It has been recently reported for lysozyme, that urea induced unfolding follows a two state model of equilibrium unfolding with no intermediate states in between the N\(\rightarrow\)D transition (Bonincontro et al, 2004). The following scheme represents the two possible unfolding pathways in GdnHCl and urea solutions for human placental cystatin.

<table>
<thead>
<tr>
<th>Native (N)</th>
<th>0.5-1.5M GdnHCl</th>
<th>Non-Native</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPC</td>
<td>Intermediate state (I)</td>
<td></td>
</tr>
<tr>
<td>Urea 8M</td>
<td>Denatured state (D)</td>
<td>GdnHCl 6M</td>
</tr>
</tbody>
</table>

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The refolding transitions determined by fluorescence measurements show that both urea and GdnHCl unfolding are greatly reversible, however, in case of GdnHCl at low concentrations the renatured protein has changed intensity compared to native and denatured forms, indicative of an intermediate state (Fig 6.7 C). The observed results could be due to combined effect of all the factors mentioned above. These different stabilizing and destabilizing effects of GdnHCl are apparently additive and lead to the complex dependence of GdnHCl concentration and the stability of protein (Rashid et al., 2005).

Understanding the conformational changes that result in a protein by various treatments would provide a powerful tool for drug design and for comprehension of cellular organization at molecular level. Cystatins have important roles to play in normal body processes owing to their cysteine proteinase inhibitory activities and it is of utmost consequence that their conformation should be stable for maximum functional activity. Thus the above observations have shed some light on the structural alterations and loss of function which result due to exposure of denaturants and thus effect the normal functioning of the protein.

II B. Acid Induced unfolding of HPC

The acid duration of HPC was studied to obtain information about the behavior of this protein at low pH conditions. The spectral parameters of fluorescence such as position, shape and intensity are dependent on the dynamic and electronic properties of the chromophore environment, hence fluorescence measurements have been extensively used to obtain information on the structural and dynamic properties of the proteins (Ghisla et al. 1974). The CD spectra of a protein in the far-UV region gives information about the conformation of polypeptide back bone and the hydrophobic dye ANS which binds to exposed hydrophobic regions of partially folded proteins is used in protein conformational studies to monitor these hydrophobic regions (Johnson and Fersht 1995, Stryer 1965).
Far UV-CD studies of acid denaturation of HPC showed no major change in ellipticity value at 222nm up to pH of 4.5, however, at pH 4.0 and below there is marked decrease in ellipticity value and a minimum value was obtained at pH 2.0. From pH 2.0 to pH 1.0 there is some increase in ellipticity value and the ANS fluorescence was increased at lower pH values with a maximum value at pH 2.0 (Fig 6.9). These two results taken together, that is, decrease in ellipticity and increase in ANS binding with decreasing pH suggest exposure of hydrophobic regions of the HPC and their availability in binding to ANS. Both ellipticity measurements at 222nm in the far UV region and ANS fluorescence show co-operative transition without formation of any intermediate states. Increase in secondary structure of HPC at pH 1.0 can be explained on the basis of anion induced folding where anions bind to the positively charged protein and decreases the number of hydrophobic patches resulting in less binding of ANS (Goto et al. 1990).

The far UV-CD spectra of human placental cystatin at pH 2.0 has almost all the features of secondary structure same as that of spectra at pH 6.0 which is the native state of protein (Fig 7.0 A), but the ellipticity value is decreased and having an α helical content of about 65% (the helical content of native state (pH 6.0) being taken as 100%). For comparison unfolding of HPC was also achieved in presence of 6M GdnHCl (Fig 7.0 A) which shows loss of secondary and tertiary structure forming a random coil structure (Rashid et al., 2005). The near UV-CD spectra of human placental cystatin is characterized by a positive peak around 280nm (Fig 7.0 B) showing significant amount of tertiary structure in this inhibitor. The absence of positive peaks in the region 250nm indicate that disulphide bonds are absent in the protein (Ramasarma et al., 1995) which supports our earlier mentioned observation of absence of any free thiol groups in this inhibitor. HPC at pH 2.0 in the near UV region has native like topology with slight decrease in ellipticity value (Fig 7.0 B). A comparison of near UV and far UV-CD spectra of acid induced state with native state shows that HPC at pH
2.0 retains lot of secondary structure as well as significant amount of tertiary structure which is close to that of native state.

The native state of HPC has fluorescence emission maxima of 345nm (Rashid et al., 2004) where as the HPC at pH 2.0 (the acid induced state) has decreased fluorescence with $\lambda_{\text{max}}$ at 335 nm (Fig 7.1). This blue shift of 10nm and decreased fluorescence indicates that the protein conformation is altered and microenvironment of aromatic amino acid has become more nonpolar. When these results are compared with the fluorescence emission spectra of HPC in presence of 6M GdnHCl which shows increase in fluorescence accompanied by a red shift of emission maxima to 350 nm compared to native state (Rashid et al. 2005), it suggested that the conformation of acid-induced state of HPC is different from both the native state at pH 6.0 and unfolded state in presence of 6M GdnHCl.

ANS binding has been widely used to detect the molten globule states of different proteins (Stryer 1965, Engelhard and Evans 1995). HPC shows large binding of ANS at PH 2.0 compared to native state accompanied by a blue shift of 30nm (Fig 7.2). The marked blue shift observed is an indication of exposed hydrophobic regions of protein present at lower pH values. Therefore, it is apparent that as compared to native state at pH 6.0 or the denatured state in presence of 6M GdnHCl the conformation of acid induced state with increased ANS fluorescence than native is different.

These results suggest that acid induced state in addition to retaining secondary and tertiary structural features also has sizeable amount of exposed hydrophobic regions. Thus there is an accumulation of a compact molten globule like intermediate at low pH possessing persistent tertiary as well as secondary structure. In the light of molten globule theory, the molten globule state of HPC at low pH conditions has similar characteristics as reported for molten globule state of other proteins (Bychkova et al. 1992) and in addition has stable native like tertiary structure. The resemblance of MG state and native state has significant bearing in understanding protein folding problems (Kataoka et al. 1993). It is important to determine the extent of tertiary structure present in MG state to understand the role of
specific side chain packing in stabilizing and specifying the MG structure. It has been shown for proteins cytochrome C, apomyoglobin and staphylococcal nuclease that small degree of side chain packing stabilizes MG states (Shiraki et al., 1995). Tertiary interactions have been reported for MG states of \( \alpha \)-lactalbumin (Shulman et al., 1997), ubiquitin (Khorasanizadeh 1996), myoglobin (Kay and Baldwin 1996) and RNase H (Raschke and Marqusse 1997). Thus it can be concluded that native like packing of core amino acids help in stabilization of molten globule state of HPC at pH 2.0 which thus has secondary and tertiary structures resembling the native state.

IIC. Effect of TFE and Methanol on refolding of acid induced state of HPC

The stabilizing effect of TFE and methanol on the acid induced molten globule state of HPC was studied to investigate its structural stability of HPC. Far UV CD showed that there is increase in ellipticity value of HPC in presence of TFE at pH 2.0. At 40 % (v/v) TFE the helical content of protein is almost same as that of the native state (at pH 6.0) (Fig 7.3A and 7.3B). The increased \( \alpha \)-helical content induced by low (20%) and moderate (50%) concentrations of TFE in denatured proteins have been reported earlier for cardiotoxin analogue I and peptide fragments of hen egg white lysozyme (Arunkumar et al. 1997, Yang et al. 1995). In case of lysozyme the helical structure induced in presence of 50% TFE was very much similar to the native state of protein. In case of HPC as the concentration of TFE increased helical content increased and maximum value was obtained at 80% concentration of TFE. The results in presence of 40% TFE state showed near UV-CD spectral features in between acid denatured state and native state however, the curve for 80% TFE shows much decreased ellipticity value indicating the denaturing effect of the alcohol at higher concentrations (Fig 7.4). Disruption of tertiary structure of protein in presence of alcohol has also been reported earlier (Kamatari et al., 1996).
Different alcohols have different helix stabilizing potential (Hirota et al. 1997, Konno et al. 2000). Thus for comparison in addition to TFE, methanol was also used to study the transition from acid denatured state to the alcohol induced state. Compared to TFE the helix inducing effect of methanol was much less as can be seen in Fig 7.5 and Fig 7.6. The respective alcohol induced transition curves of HPC for TFE and methanol showed that in case of TFE there is marked increase in the ellipticity as shown in far UV-CD spectra of HPC (at pH 2.0) with the increase in concentration of these alcohols whereas same effect is not observed in presence of methanol. As the concentration of methanol increases to 50% there is not much variation in far UV-CD intensity and with further increase in concentration of methanol the intensity remained constant. Therefore it can be concluded that TFE has much higher helix inducing ability as compared to Methanol.

Effect of TFE on acid-induced state of HPC shows that alcohols do not induce major structural changes but significantly modify the unfolded protein by inducing α-helical structures (Fig 7.3, 7.4 and 7.5). Thus, TFE while inducing secondary structural features leads to the disruption of tertiary structure (Fig 7.4). Therefore, TFE induced state at higher concentrations (80%) with significant helical structure and disrupted tertiary structure has some molten globule like characteristics but it is significantly different from the compact molten globule state at pH 2.0 which has stable hydrophobic interactions and more resemblance to native state in terms of secondary and tertiary structural features.

The TFE induced molten globule like state can be referred to as non-interacting open helical structure as reported elsewhere (Thomas and Dill 1993, Hagihara et al. 1994, Nishii et al. 1994) in which the interactions between the helical segments are weak. The decreased hydrophobic interactions among helical segments explain the presence of disrupted tertiary structure in TFE induced state of HPC, although this conformation has high secondary structural content (or increased helicity) due to stabilization of polar interactions or hydrogen bonds in the presence of TFE.
The transition curves in Fig 7.7 showed that increase in alcohol concentration leads to decrease in ANS fluorescence showing non availability of hydrophobic clusters for binding in presence of alcohols. The decrease in ANS fluorescence is more in presence of TFE than methanol. Decrease in ANS fluorescence has also been reported earlier (Kamatari et al. 1996). The acid induced state of HPC in the presence of 80 % TFE showed decreased fluorescence emission as compared to native state with an emission maxima of 370nm (Fig 7.1). This red shift suggests disordered tertiary structure in the presence of increasing alcohol concentration. The fluorescence intensity shown by ANS bound to HPC at pH 2.0 in the presence of 80% TFE is very less with λmax of 510nm which is characteristic of free ANS in water suggesting absence of any hydrophobic clusters under these conditions (Fig 7.2).

From these results it may be inferred that the addition of alcohol to the acid denatured HPC induces a state characterized by more helical structure and highly internalized hydrophobic regions. Halogenated alcohol, that is, TFE has strong effectiveness in conversion of acid denatured state to alcohol induced state, suggesting that fluorine-atom is important for enhancing the effect of alcohol. It has been shown earlier that co-operative α helix formation in α lactalbumin and melittin induced by hexa fluoroisopropanol, a compound with six fluorine atoms is more rapid in inducing this type of change than TFE which contains only three fluorine atoms (Hirota and Goto 1999).

Our results in the present study may be summarized as (a) formation of acid induced molten globule (MG) state at pH 2.0 having significant secondary and tertiary interactions which have resemblance to native state and has exposed hydrophobic regions (b) effect of TFE compared to methanol is more pronounced in conversion of acid denatured state of HPC to alcohol induced state, which is characterized by increased helical content, disrupted tertiary structure and absence of hydrophobic clusters.
II D. Effect of polyols and salts on refolding of acid induced state of HPC

Both polyols and salts have stabilizing effect on proteins. The ability of co-solvents to stabilize proteins has been attributed to the preferential hydration of proteins (Nolting et al., 1995; Privalov 1996), which means that co-solvents do not interact directly with the protein molecule, i.e. they preferentially solubilizes in the bulk water. In a triphasic system consisting of water, protein and co-solvent (a stabilizer) – the stabilizer is excluded from the vicinal water that composes the solvent layer of protein. As a result the protein becomes preferentially hydrated, but the radius of the solvation layer and the apparent volume of the protein decreases in a phenomenon that leads to more stable conformation (Felix et al., 1999; Lopes et al., 1999). Kamiyama et al., (1999) has reported molten globule formation in presence of polyols for horse Cytochrome C. In the present study polyols, glycerol and sorbitol showed different degree of stabilizing effects on acid denatured state of HPC. There was a considerable increase in ellipticity values of HPC with the increase in glycerol concentration as shown by far UV-CD measurements (Fig 7.8). At 80 % (v/v) glycerol the far UV-CD spectra of HPC at pH 2.0 showed that the protein has gained highly stabilized structure like that of native state. This glycerol induced state has 98% of α helical content compared to the value of 65% for acid induced state of HPC (α helical content of native state at pH 6.0 taken as 100%). Another polyol used in the study that is sorbitol had less structure stabilizing effect on acid induced state of HPC as compared to that of glycerol (Fig 7.8 B and C). This clearly shows that glycerol has higher protein stabilizing potential.

The polyol induced transition which was more effective for glycerol than sorbitol can be explained on the basis that HPC is preferentially hydrated as indicated above, predominantly due to unfavorable interaction of polyols with non-polar amino acid residues resulting in exclusion of polyols from the protein surface while being retained in the bulk. Thus glycerol stabilizes the protein structure by strengthening hydrophobic interactions and by overcoming the electrostatic interactions between the charged
residues (Kamiyama et al., 1999; Gekko and Ito 1990; Mason et al., 2003). In case of glycerol-induced state at 80% concentration fluorescence intensity is almost same as that of native state but with a 10nm blue shift indicating that the aromatic amino acid residues still have non-polar environment where as for 80% sorbitol-induced state fluorescence intensity was decreased compared to that of pH 2.0 state suggesting the presence of lesser amounts of tertiary folds in the protein (Fig 7.9).

The acid induced state of HPC in presence of 80% glycerol also showed ANS fluorescence with emission maxima of 480nm indicating the presence of sizeable amount of hydrophobic clusters but ANS binding was less than that of the pH 2.0 state where ANS binding is maximum (Fig 8.0). These results show that glycerol induced state has different conformation as compared to that of sorbitol induced state which showed minimal ANS binding. The glycerol induced state showed conformation close to the tertiary structure of protein in the native state as there is some ordering of hydrophobic regions indicated by decreased ANS binding as compared to the acid-induced state alone.

In the present study in addition to polyols, three salts (MgSO₄, Na₂SO₄, and MgCl₂) are considered for their protein stability effects. The far UV CD studies of acid induced state of HPC in the presence of these salts showed that both the sulphate salts are less effective in stabilizing the protein (Fig 8.1 A and B). However, presence of MgCl₂ showed more pronounced stabilizing effect on HPC at pH 2.0 as compared to that of sulphate salts. At 3M MgCl₂ concentration spectral features showed resemblance to the native state of protein (Fig 8.1 C and D). The maximum fluorescence emission of HPC at pH 2.0 is increased in presence of both the sulphate salts (Fig. 8.2) when compared to that of native (pH .60) and acid-induced states (pH 2.0) alone. The large increase in fluorescence observed for MgSO₄ than Na₂SO₄ suggests a change in the environment of aromatic amino acid residues with a decreased tertiary structure. The spectra for MgCl₂ has same intensity as that of acid-induced state but shows red shift of 5nm which indicates the changed tertiary structural features when compared
to acid-induced state. The ANS binding studies show least ANS binding in the presence of sulphate salts whereas in presence of MgCl₂ ANS binding is more than that of native state at pH 6.0 although being less than the acid induced state with a 30nm red shift (Fig 8.3). The ANS binding in case of both sulphate salts is less than that of the native state showing least presence of hydrophobic clusters. The intrinsic fluorescence and ANS binding studies show that MgCl₂-induced state has more stable conformation as compared to the structures induced by sulphate salts and it lies somewhere in between the native and acid-induced state.

The CD spectrum in the near UV region has been used to probe the asymmetry in the environment of proteins aromatic amino acid residues (Kuwajima 1989; Dryden and Weir 1991). The near UV-CD spectral studies show that HPC at pH 2.0 in presence of 80% glycerol and 3M MgCl₂ has ellipticity values higher than that of acid induced state alone, however less than that of the native state of protein at pH 6.0 (Fig 8.4). The increase in tertiary structure as shown by near UV-CD spectra is more for glycerol induced state than the MgCl₂ state. Although the ellipticity values obtained in the presence of 80% glycerol was less than the ellipticity of native HPC, it certainly shows the attainment of tertiary structure close to native state in presence of glycerol.

The protein stability in the presence of salts and increased induction of helical structure can be explained on the basis of ‘Salting out’ effects of these salts as reported earlier (Arakawa and Timasheff 1984; Arakawa et al., 1990 (a), 1990 (b)). In order to explain further the differential effects of salts on protein stabilization, experiments in the wide concentration range are necessary to discriminate the ‘Salting out’ effect from the preferential salt binding (salting in) effects. The MgCl₂ induced refolding of HPC is accompanied by induction of significant secondary and tertiary structure and overall increased compactness of the protein molecule compared to the sulphate salts. At low pH conditions there is net positive charge on the proteins, it is more likely that it is the anions that are the key components in the action of salts. One indication that the effects involve a significant direct
ion interaction with the protein is that although both sulphate and chloride have stabilizing effects on the protein, sulphate is a known Kosmotropic (stabilizing) agent for protein, however, chloride is neutral in this regard (Collins and Washabaugh 1985). Thus, it is most likely that the effects are due to specific interaction of ions with the protein, in addition to preferential hydration of protein in the presence of salts. Thus chloride ions at high concentration, 3M used in this case, binds to the positively charged HPC (at pH 2.0) and stabilizes the protein on account of specific ion interactions. The ion-induced effects on the water structure at high salt concentration could in turn effect the hydrophobic interactions within the protein (Kella and Kinsella 1988). All these effects taken together explain differential effects of salts in protein stabilization.

The overall results suggest that the acid denatured state of HPC in presence of polyols and salts refolds in to a structure which lies in between the native and acid denatured conformation. Among polyols, glycerol showed more protein stabilizing effect as compared to sorbitol. The glycerol-induced state at 80% concentration has some molten globule like characteristics. It has often been claimed that the stability of molten globule state is determined by balancing between the hydrophobic interactions and the electrostatic repulsive forces since studies on the amino acid solubility and preferential solvent interaction of protein in polyol-water mixtures clearly demonstrated that the main driving force of protein stabilization by polyols is the enhancement of hydrophobic interactions overcoming the electrostatic repulsion between the charged residues (Kamiyama et al., 1999; Goto et al., 1990). Figs. 7.8A, 7.9 and 8.0 reveal following features of HPC at pH 2.0 in presence of glycerol (80%), native like secondary structural features as shown by far UV-CD, increased binding of ANS compared to native state (pH 6.0) although less than that of acid denatured state (pH 2.0), blue shift of intrinsic fluorescence measurement indicating apolar environment of aromatic amino acid residues, slightly increased ellipticity as compared to acid denatured state in near UV region as shown in Fig 8.4. Thus, with these features glycerol induced state (80% concentration) is
different from acid denatured state which has a molten globule conformation. On the basis of above considerations this glycerol induced state can be regarded as a state in between native and MG state at pH 2.0. The decreased binding of ANS observed in the glycerol induced state can be explained as decrease of ANS binding sites or an increased ordering of tertiary structure making ANS binding sites more inaccessible.

ANS binds to hydrophobic patches on protein (Stryer 1965). In native state ANS binding sites are buried deep in the protein interior, increased accessibility of ANS to these sites in the molten globule states results from the loose packing of residues in this conformation (Semisotnov et al., 1991). Since both intrinsic fluorescence and near UV-CD show glycerol-induced state resembling to native state, it suggests ordering of tertiary structure. Similar is the case in the presence of MgCl₂ (3M) where HPC has conformation in between the native and acid induced state as can be seen in Fig 8.1C, Fig 8.2, Fig 8.3 and Fig 8.4. Thus, on the basis of these studies it can be concluded that both polyol (80% glycerol) and salt (3M MgCl₂) induced states are different from molten globule states at pH 2.0 and have compactness lying in between native and MG state. As the degree of preferential exclusion of a co-solvent is directly proportional to the protein surface area, the system under a particular set of conditions favors the protein state with the smallest area (Kendrick et al., 1997) and preferential exclusion of co-solvents leads to increased compactness of protein compared to native state, especially in view of the unfavorable interactions between water and the polypeptide backbone (Liu and Bolen 1995). This explains the mid-way conformation lying in between the native and molten globule state (at pH 2.0), of the co-solvent induced states of HPC.

In conclusion our results showed that human placental cystatin (HPC) is a type 1 cystatin similar in some properties to type 2 mammalian cystatins. The investigations have shed some light on the structure of this inhibitor from an important human tissue, which certainly will add to the scientific information available about these inhibitors and it will help in understanding cystatins in a wider perspective.