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Effect of Polyols and Salts on the Acid-Induced State of Human Placental Cystatin

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Abstract—Polyols (glycerol and sorbitol) and salts (magnesium sulfate, sodium sulfate, and magnesium chloride) have been used to study the refolding of the acid-induced state of human placental cystatin (HPC), which is a low molecular weight (12,500 daltons) thiol proteinase inhibitor, in terms of CD spectroscopy, binding of hydrophobic dye 1-anilinonaphthalene-8-sulfonic acid (ANS), and intrinsic fluorescence measurements. The helical content of acid-denatured HPC increased with increase in glycerol concentration (0-80%). At 80% glycerol concentration, the secondary structural features observed in the far UV-CD region are similar to those of the native state (pH 6.0). The intrinsic fluorescence and near UV-CD studies showed that this 80% glycerol-induced state has a significant amount of tertiary structure with decreased ANS binding compared to the acid-denatured state. It was found that glycerol is more effective in stabilizing the acid-denatured state of HPC as compared to sorbitol. Among salts the stability effect was more for $MgCl_2$ (used up to concentration of 3 M) compared to $MgSO_4$ and Na_2SO_4 (used up to the concentration of 1.5 M due to restricted solubility of HPC at higher sulfate salt concentrations) as determined by CD studies and fluorescence measurements, which showed secondary and tertiary structural resemblance of this $MgCl_2$-induced state close to native state and showed overall spectral features in between the native state and the acid-denatured state. This $MgCl_2$ (3 M)-induced state showed decreased ANS fluorescence as compared to the acid-denatured state but more than that of the native state. The results taken together suggest that the acid-denatured state of HPC in the presence of 80% glycerol or 3 M $MgCl_2$ has a conformation in between that of the native state (pH 6.0) and the acid-induced state at pH 2.0.

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The stability of proteins in solution is a major concern of biologists and pharmacologists. The more in-depth knowledge of protein denaturation and refolding is central in selection of a particular solvent for protein stabilization. The mechanism by which proteins fold from a denatured structureless state to their biologically active form is an intricate process [1, 2]. Recent technological progress especially in the field of biophysical studies has helped to show the presence of intermediate states during the folding and unfolding pathways [3, 4]. These intermediate states or partially unfolded states have been studied for several proteins [5, 6], and characterization of these conformational transitions has helped in understanding the phenomenon of protein folding [7, 8]. It has been shown that the amount of tertiary structure and the compactness of an intermediate state vary from one protein to another [9, 10]. These intermediate states have structures either close to the native state or unfolded state depending upon the protein being studied and the experimental conditions used. Characterization of such states can be of utmost importance in further understanding the protein folding phenomenon. There are various ways of studying protein stabilization, for example, denaturation in the presence of guanidine hydrochloride (GdnHCl) or urea and acid denaturation.

We have earlier reported unfolding and inactivation of human placental cystatin (HPC) in the presence of denaturants urea and GdnHCl [11]. The urea-induced

Abbreviations: ANS) 1-anilinonaphthalene-8-sulfonic acid; CD) circular dichroism; GdnHCl) guanidine hydrochloride; HPC) human placental cystatin; MG) molten globule; MRE) mean residue ellipticity.

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denaturation (in concentration range of 1-8 M urea) of HPC followed the two-state rule in which the native-to-denatured-state transition occurs in a single step, whereas in case of GdnHCl-induced denaturation (in concentration range of 1-6 M), intermediate or non-native states are observed at lower concentration of denaturant (0.5-1.5 M). HPC was found to be completely denatured at 8 M urea or 6 M GdnHCl concentration [11].

The acid-induced state of various proteins show that it is less unfolded compared to the completely unfolded form obtained in 6 M GdnHCl and 9 M urea [12, 13]. In acid denaturation the major driving force is intramolecular charge repulsion, which may fail to overcome the interactions favoring folding such as hydrophobic forces, disulfide bonds, salt bridges, and metal ion–protein interactions [5]. Under conditions of low pH, it has been shown that several proteins have compact structure with native-like secondary structure and largely disordered tertiary structure, that is, the characteristics of the molten globule (MG) state which is considered to be a general intermediate in the protein folding pathway of proteins [10, 14]. Polyols and salts have been used for stabilization of proteins in various studies [15]. Polyols are one of the groups of osmolytes that are able to stabilize proteins [16, 17]. The ability of cosolvents to stabilize proteins has been attributed to the preferential hydration of proteins [1, 9], which means that cosolvents do not interact directly with the protein molecule that is preferentially solubilizing in the bulk water. In a triphasic system consisting of water, protein, and cosolvent (a stabilizer), the stabilizer is excluded from the vicinal water that composes the solvent layer of the protein. As a result, the protein becomes preferentially hydrated, but the radius of the solvation layer and the apparent volume of the protein decrease in a phenomenon that leads to more stable conformation [16-18]. Kamiyama et al. [19] reported molten globule formation in the presence of polyols for horse cytochrome *c*. They have shown that polyols can stabilize the MG state of the protein through enhanced hydrophobic interactions overcoming the electrostatic repulsion between the charged residues.

The aim of the present investigation is to show effects of polyols (glycerol and sorbitol), sulfate salts ($MgSO_4$ and Na_2SO_4), and chloride salt ($MgCl_2$) on the structure of the acid-induced state of HPC. In normal pregnancy and various pregnancy-related disorders, the placenta plays a highly significant role [20]. Lysosomes present in the cells of the placenta take part in intracellular degradation of proteins [21], and the activity of lysosomal cysteine proteinases (cathepsins B, H, L, and S) is controlled by cystatins present in the placenta [22]. Cystatins are noncovalent competitive inhibitors of cysteine proteases [23, 24]. They are present in a variety of tissues and body fluids of humans and animals. They are also present in microorganisms and plants [25, 26]. Cystatins are associated with various pathological conditions such as rheumatoid arthritis [27], metastasizing cancer [28], renal failure [29], osteoporosis [30], and septic shock [31] resulting due to imbalance between endogenous inhibitors and cysteine proteases. There are three subtypes of the cystatin superfamily—family I cystatins with molecular weight of 11,000-12,000 daltons lacking disulfide bonds and carbohydrate content; family II cystatins having two disulfide bonds and carbohydrate content with molecular weight in the range of 13,000-14,000 daltons, and family III cystatins or kininogens which are high molecular weight proteins present only in mammalian plasma [23]. HPC used in this study was purified from human placenta by the method of Rashid et al. [32]. It is a low molecular weight protein (12,500 daltons) with no disulfide bonds and carbohydrate content. Owing to these properties and various other properties, which we have reported earlier, it can be placed in family I cystatins [33]. As already mentioned, HPC plays a vital role in control of unwanted proteolysis in the placental tissue, so studies were carried out to analyze the conformational alterations in the structure of this protein. Any change in the structure can affect the normal functioning of this protein and lead to uncontrolled intracellular degradation of proteins. Folding studies on HPC may prove useful in understanding of the pathway by which the protein attains its native conformation and various conditions that can be employed to refold denatured protein back to its native state.

MATERIALS AND METHODS

Materials. 1-Anilinonaphthalene-8-sulfonic acid (ANS), glycerol, sorbitol, and Sephadex $G_{50\text{-}80}$ were obtained from Sigma (USA). All other chemicals used were of the highest analytical grade.

Sample preparation. Cystatin was isolated and purified from human placenta in high yield and fold purification by the method of Rashid et al. [32]. The purified human placental cystatin gave a single band on 12.5% polyacrylamide gel electrophoresis (PAGE) under native, reducing, and non-reducing conditions as reported earlier [32].

Spectrophotometric measurements. The protein concentration was determined by the method of Lowry et al. [34]. A stock solution of ANS in distilled water was prepared and concentration determined using an extinction coefficient of 5000 $M^{-1}\cdot cm^{-1}$ at 350 nm [35]. The molar ratio of ANS to protein was 1 : 60.

Conformational transitions. Acid-induced unfolding of native HPC was carried out by incubating the protein with buffers of pH 2.0 (glycine-HCl buffer), sodium acetate (pH 3.0-5.0), and sodium phosphate (pH 6.0-7.0) at 4°C and allowed to equilibrate for 4 h before taking spectrophotometric measurements. Denaturation of HPC at pH 2.0 in the presence of GdnHCl was studied by

far UV-CD. Increasing amounts (1-6 M) of GdnHCl were added to a fixed amount of protein (23.2 μM) and allowed to equilibrate before taking CD measurements at 222 nm. Mean residue ellipticity (MRE) values were calculated according to Chen et al. [36] and plotted against denaturant concentration. Cosolvent-containing solutions were freshly prepared for each study with a volumetric adjustment of concentrations. Glycerol and sorbitol were used in concentration range up to 80% (v/v), whereas salt concentrations used were restricted by the maximum stability of HPC in each salt solution. Sulfate salts were used up to the concentration of 1.5 M, whereas $MgCl_2$ was used up to 3 M concentration for the respective protein stability studies.

Circular dichroism measurements. Circular dichroism (CD) measurements were carried out at 25°C on a Jasco model J-20 spectropolarimeter using a SEKONIC XY plotter (model SPL-430A) with thermostatically controlled cell holder attached to a Neslab model RTE 110 water bath with an accuracy of ±0.10°C. The instrument was calibrated with d-10-camphorsulfonic acid. The spectra were recorded with a scan speed of 20 nm/min and with a response time of 4 sec. Each spectrum was recorded as an average of five scans. The concentration of HPC was 23.2 μM for far UV-CD in the wavelength region 200-250 nm in a 0.1-cm path length cuvette and 80 μM for near UV-CD in the wavelength region 250-300 nm in a 1-cm pathlength cuvette. The CD results are expressed as MRE in deg·cm^2·dmol^{-1} using the equation given by Chen et al. [36]:

$$\text{MRE}\ (\theta) = \theta_{obs}\ (\text{mdeg})/10{\cdot}n{\cdot}l{\cdot}C_p,$$

where θ_{obs} is the CD in millidegrees, n is the mean residue weight (110), l is the pathlength of the cell (cm), and C_p is the mole fraction.

Fluorescence measurements. Fluorescence was measured on a Shimadzu model RF-540 equipped spectrofluorimeter with a DR-3 data recorder at 25°C. The fluorescence was recorded in the wavelength region 300-400 nm while exciting the protein solution at 280 nm for intrinsic fluorescence measurements (the protein was excited to observe collective effects of aromatic amino acids with the major effect being of tryptophan residues). The spectral parameters of tryptophan fluorescence such as position, shape, and intensity are dependent on the dynamic and electronic properties of the chromophore environment; hence, steady state fluorescence has been extensively used to obtain information on the structural and dynamic properties of proteins [37]. The slits were set at 5 nm for excitation and emission and the pathlength of the sample was 1 cm. The protein concentration used was 1 μM. Binding of ANS to HPC under various conditions was studied by exciting the dye at 380 nm, and the emission spectra were recorded from 400-600 nm wavelength range with a 10-nm slit width for excitation and emission.

RESULTS AND DISCUSSION

The CD spectra of a protein in the far UV region give information about the conformation of the polypeptide backbone. Figure 1 shows that as the pH decreases from pH 7.0 to 2.0, there is no major change in ellipticity value at 222 nm up to pH of 4.5, but at pH 4.0 and below there is a marked decrease in ellipticity value reaching a minimum value at pH 2.0. In this transition to pH 2.0, there is loss of 34.5% of the secondary structure as compared to that of the native conformation (taken to be having 100% secondary structure). Ellipticity measurements at 222 nm in the far UV region showed a weakly cooperative transition without formation of any intermediate states. Denaturation of HPC at pH 2.0 in presence of varying amounts of GdnHCl was also investigated by far UV-CD. As seen in Fig. 2, GdnHCl induces further unfolding of the residual secondary structure detected in HPC at pH 2.0. Such a sigmoidal transition has also been reported for α-lactalbumin at acid pH [38].

Both polyols and salts have a stabilizing effect on proteins with strong preferential hydration effect [39]. Figure 3 shows the far-UV CD spectra of HPC in its native state (pH 6.0) and acid-induced state (pH 2.0) in the absence and presence of increasing concentration of glycerol (20-80% v/v). The native state of HPC has a significant amount of secondary structure and shows minima at 222 and 208 nm. It has α-helical content of 21.08% as determined by the method of Chen et al. [36], as has been reported earlier [33]. The acid-induced state has 65% of native secondary structure with decreased ellipticity compared to the native conformation. As the glycerol concentration is increased up to 80%, there is increase in the ellipticity values compared to that of the acid-induced state. At 20% glycerol, there is no marked change in ellip-

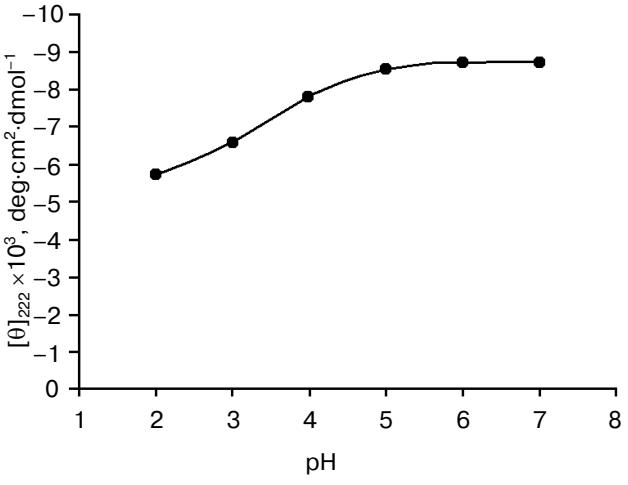

Fig. 1. The pH dependence of CD spectra of HPC at 25°C. MRE values (•) were monitored at 222 nm. The concentration of protein used was 23.2 μM.

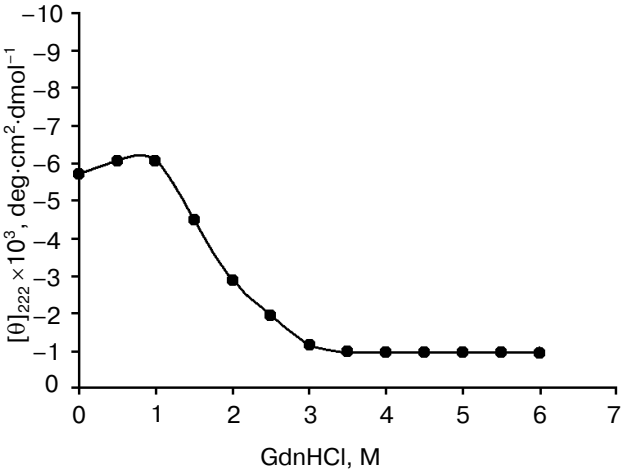

Fig. 2. The GdnHCl-induced transition of HPC at pH 2.0 as monitored by far UV-CD changes at 222 nm. Increasing amounts of GdnHCl (1-6 M) were added to a fixed amount of protein (23.2 μM).

ticity and the stabilization or the structure-inducing effect is much less. As the concentration of glycerol is increased from 40-80%, there is a large increase in ellipticity values, and at 80% glycerol concentration the structure is almost the same as that of the native state. This glycerol-induced state has 98% of the native state α-helical content. Figure 3 (inset) shows the effect of increasing concentration of sorbitol on the acid-induced state of HPC. As can be seen from this figure, although there is an increase in ellipticity values of the protein with the increase in sorbitol concentration from 20-80%, the effect is much less as compared to that of glycerol. This clearly shows that glycerol has higher protein-stabilizing potential when compared to that of sorbitol. The polyol-induced transition, which was more effective for glycerol than sorbitol, can be explained on the basis that HPC is preferentially hydrated, predominantly due to unfavorable interaction of polyols with nonpolar 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 [18, 40, 41].

Figure 4a shows the fluorescence emission spectra of HPC in the native state (pH 6.0) as well as in the acid-induced state (pH 2.0) in the absence and presence of 80% glycerol and 80% sorbitol. The fluorescence emission spectrum of HPC under native conditions (pH 6.0) has a λ_{max} of 345 nm as reported earlier [11, 33]. The acid-induced state has a slightly decreased fluorescence and shows a blue shift of 10 nm indicating that the protein conformation is being altered and the microenvironment of aromatic amino acids is becoming more nonpolar. The fluorescence spectra of the glycerol-induced state at 80% concentration show intensity almost the same as that of the native state but with a 10-nm blue shift indicating that the aromatic amino acid residues still have nonpolar environment, whereas when 80% sorbitol was added to the acid-induced state it showed decreased intensity compared to that of the pH 2.0 state suggesting the presence of a less stable structure in the protein under this condition.

Binding of ANS to protein has been extensively used to detect the presence of hydrophobic clusters on the surface of proteins [42]. Figure 4b shows ANS binding to various states of HPC. The acid-induced state at pH 2.0 shows the maximum binding of ANS, suggesting maximum exposure of hydrophobic clusters. These results suggest that the acid-induced state in addition to retaining secondary and tertiary structural features also has a sizeable amount of exposed hydrophobic regions. The glycerol-induced state at 80% (v/v) glycerol concentration also shows ANS binding but to a lesser extent compared to that of the acid-induced state. Both have emission maxima of 480 nm suggesting that the glycerol-induced state has a sizeable amount of exposed hydrophobic clusters, whereas at 80% sorbitol concentration ANS fluorescence is very low. These results show that the glycerol-induced state has different conformation as compared to that of the sorbitol-induced state and the protein in the

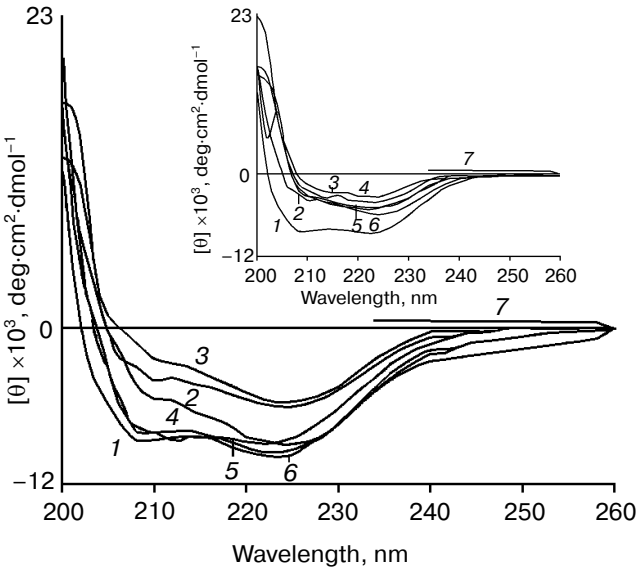

Fig. 3. Effect of polyols on the secondary structure of HPC. The figure shows far UV-CD spectra of HPC at pH 6.0 (*1*), pH 2.0 (*2*), and as function of glycerol concentration in 20 mM glycine-HCl buffer, pH 2.0, at 25°C. The concentration of glycerol is 20 (*3*), 40 (*4*), 60 (*5*), and 80% (v/v) (*6*), respectively; *7*) in presence of 6 M GdnHCl. The concentration of HPC was 23.2 μM and pathlength was 0.1 cm. Inset shows far UV-CD spectra of HPC under the same conditions as above only that glycerol has been replaced by sorbitol.

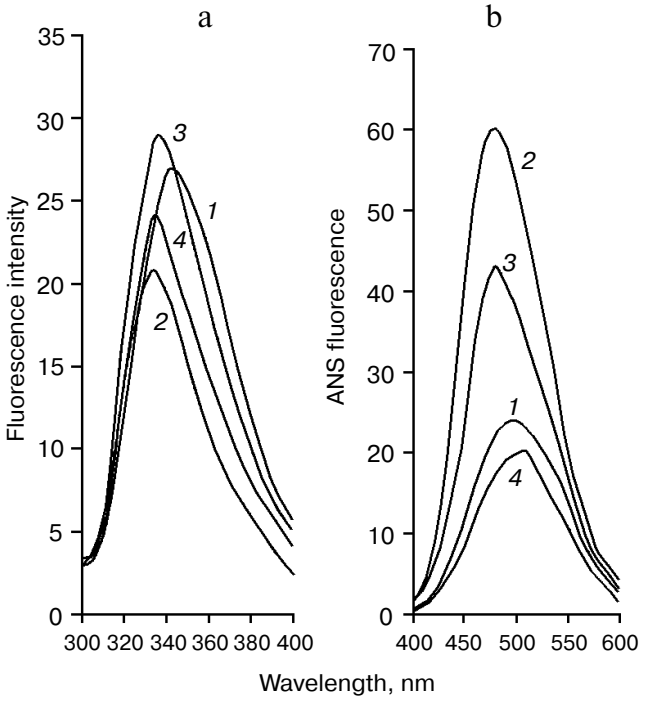

Fig. 4. a) Intrinsic fluorescence analysis of HPC at pH 6.0 (*1*) and 2.0 (*2*) in the absence and presence of 80% (v/v) glycerol (*3*) and 80% (v/v) sorbitol (*4*). The concentration of HPC was 1 μM. HPC was preincubated for 4 h at 25°C in buffer of pH 6.0 for the native and pH 2.0 for the acid-induced state and at pH 2.0 in presence of polyols for the respective fluorescence measurements. Fluorescence was measured with an excitation wavelength of 280 nm in the emission range of 300-400 nm with a slit width of 5 nm. b) Fluorescence emission spectra of ANS bound to HPC under the same conditions as in Fig. 4a. The ANS-to-protein molar ratio was 1 : 60. ANS fluorescence was measured with an excitation wavelength of 380 nm in the emission range of 400-600 nm with a slit width of 10 nm.

latter case is less stable and with the least resemblance to the native state. Thus, the glycerol-induced state has a conformation which is close to the tertiary structure of the protein in the native state.

The preferential hydration effect shown by salts is the so-called "salting out" effect. Protein stabilizing effects have been earlier reported for sulfate salts [43]. In the present study, three salts ($MgSO_4$, Na_2SO_4, and $MgCl_2$) are considered for their protein stability effects. Figure 5a shows far UV-CD spectra of HPC in the native state as well as in the acid-induced state in the absence and presence of 1 and 1.5 M $MgSO_4$. It can be seen from the figure that these salt concentrations have no profound effect on the refolding of HPC denatured under low pH conditions. As the concentration of $MgSO_4$ increases there is decrease in ellipticity values, although the spectra show some secondary structural features in the form of minima at 208 and 222 nm for 1 M salt concentration, whereas at 1.5 M salt concentration the peak at 208 nm is absent. It should be noted that the maximum concentration of salt

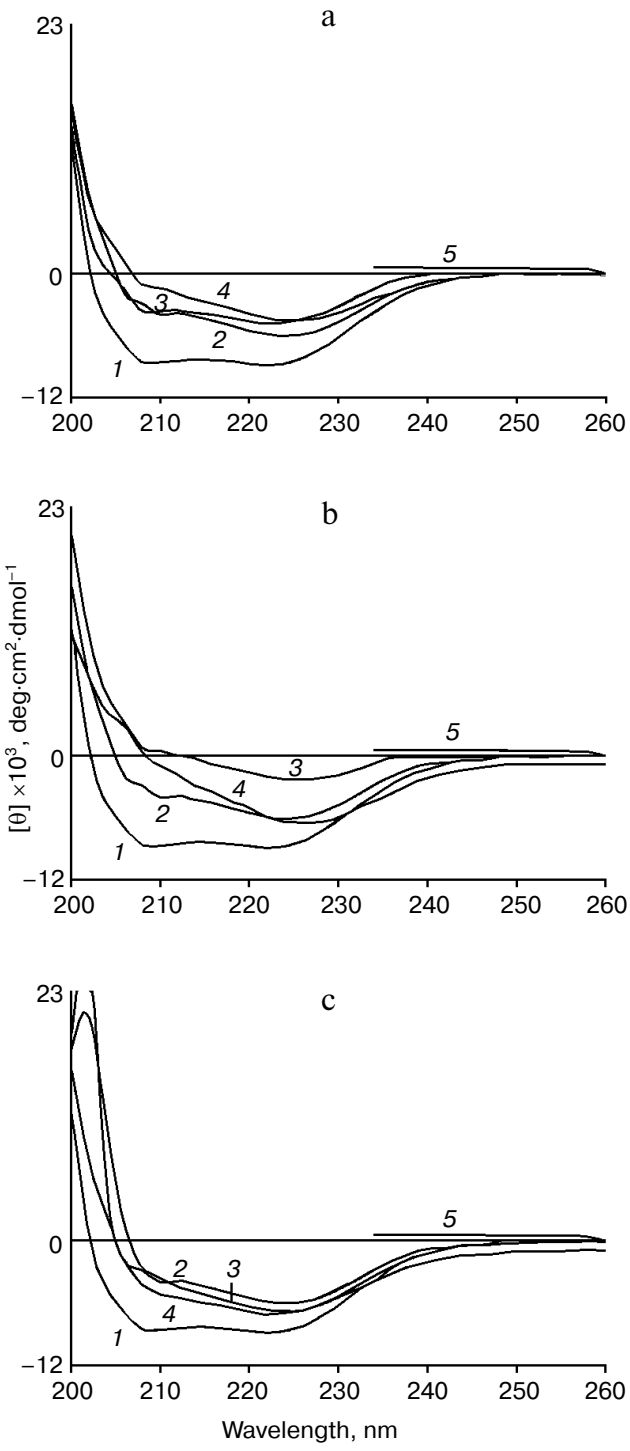

Fig. 5. Effect of salts on the secondary structure of HPC. a) Far UV-CD spectra of HPC at pH 6.0 (*1*), at 6 M GdnHCl (*5*), at pH 2.0 (*2*), and as function of $MgSO_4$ concentration in 20 mM glycine-HCl buffer, pH 2.0, at 25°C. The concentration of $MgSO_4$ used is 1 M (*3*) and 1.5 M (*4*). The concentration of HPC was 23.2 μM and the pathlength was 0.1 cm. b) Far UV-CD spectra of HPC under the same conditions as in Fig. 5a only that $MgSO_4$ has been replaced by Na_2SO_4. c) Far UV-CD spectra of HPC under the same conditions as in Fig. 5a only that $MgSO_4$ has been replaced by $MgCl_2$. The concentration of $MgCl_2$ used is 2 M (*3*) and 3 M (*4*).

used in this study to monitor ellipticity was the one in which the solutions were clear, and higher concentrations of salt led to precipitation of protein. Figure 5b shows far UV-CD spectra of HPC in the native state as well as in the acid-induced state in the absence and presence of 1 and 1.5 M Na_2SO_4. At both 1 and 1.5 M concentrations of this salt HPC shows decreased ellipticity and loss of minima at 208 nm, with a broad peak at 222 nm. The third salt used in this study, $MgCl_2$, has increased protein stabilizing effect as shown in Fig. 5c. The figure indicates that the ellipticity values in the presence of both 2 and 3 M salt concentrations are increased as compared to that of the acid-induced state of HPC. At 3 M salt concentration, the spectral features resemble those of the native state.

Fluorescence emission measurements in Fig. 6a show that the fluorescence intensity in the presence of both the sulfate salts is increased as compared to that of the native and acid-induced states. The larger increase in fluorescence observed for $MgSO_4$ than Na_2SO_4 suggests a change in the environment of aromatic amino acid residues with a decreased tertiary structure. The spectra for $MgCl_2$ have the same intensity as that of the acid-induced state but show red shift of 5 nm, which indicates changed tertiary structural features when compared to the acid-induced state. ANS binding experiments in Fig. 6b show that these salts have different effects on the ANS binding properties of HPC at pH 2.0. The $MgCl_2$-induced state shows increased binding of ANS compared to the native state at pH 6.0, but the λ_{max} at 510 nm showed a red shift of 30 nm compared to the acid-induced state. The ANS binding in the case of both sulfate salts is less than that of the native state, showing the least presence of hydrophobic clusters. The intrinsic fluorescence and ANS binding studies show that the $MgCl_2$-induced state has more stable conformation as compared to the structures induced by sulfate salts and it lies somewhere in between the native and acid-induced states.

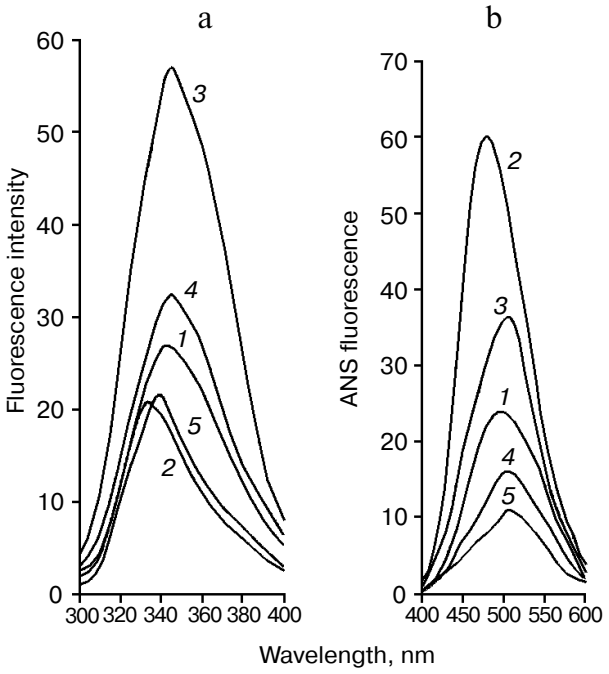

Fig. 6. a) Intrinsic fluorescence analysis of HPC at pH 6.0 (*1*) and 2.0 (*2*) in the absence and presence of 1.5 M $MgSO_4$ (*3*), 1.5 M Na_2SO_4 (*4*), and 3 M $MgCl_2$ (*5*). The concentration of HPC was 1 μM. HPC was preincubated for 4 h at 25°C in buffer of pH 6.0 for the native state and pH 2.0 for the acid-induced state and at pH 2.0 in the presence of salts for the respective fluorescence measurements. Fluorescence was measured with an excitation wavelength of 280 nm in the emission range of 300-400 nm with a slit width of 5 nm. b) Fluorescence emission spectra of ANS bound to HPC under the same conditions as in Fig. 6a: at pH 6.0 (*1*) and 2.0 (*2*) in the absence and presence of 3 M $MgCl_2$ (*3*), 1.5 M $MgSO_4$ (*4*), and 1.5 M Na_2SO_4 (*5*). The ANS-to-protein molar ratio was 1 : 60. ANS fluorescence was measured with an excitation wavelength of 380 nm in the emission range of 400-600 nm with a slit width of 10 nm.

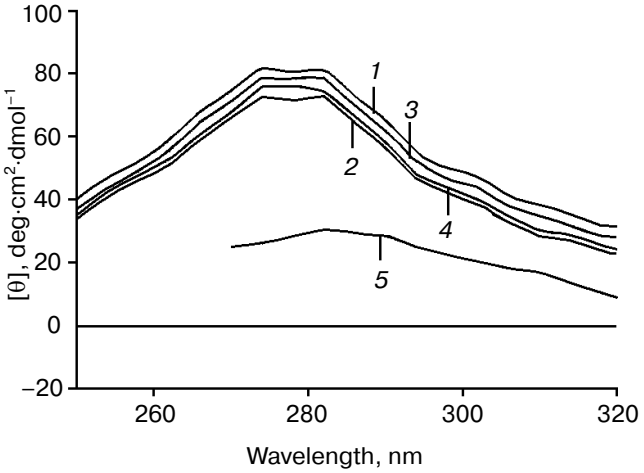

Fig. 7. Tertiary structure analysis of HPC showing near UV-CD spectra of the protein at pH 6.0 (*1*) alone and in the presence of 6 M GdnHCl (*5*), at pH 2.0 in the absence (*2*) and in the presence of 80% (v/v) glycerol (*3*) and 3 M $MgCl_2$ (*4*). The concentration of HPC for near UV-CD was 80 μM and path length was 1 cm.

The CD spectra in the near UV region have been used to probe the asymmetry in the environment of aromatic amino acid residues of the protein [44]. Figure 7 shows the near UV-CD spectra in the 250-300 nm range of native and acid-denatured HPC both in the absence and presence of 80% (v/v) glycerol and 3 M $MgCl_2$. As can be seen from the figure, the near UV-CD spectra of native HPC is characterized by a peak at 280 nm suggesting the presence of tertiary structure in the protein. Near UV-CD spectra of the acid-induced state of HPC also showed these characteristics but the ellipticity values are reduced, showing change in protein tertiary structure. In the presence of 80% glycerol and 3 M $MgCl_2$, there was an increase in ellipticity value compared to that of the

acid-induced state, the effect being more for glycerol. Although the ellipticity values obtained in the presence of 80% glycerol were less than the ellipticity of native HPC, it certainly shows the attainment of tertiary structure close to the native state in the presence of glycerol.

The protein stability at pH 2.0 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 [43]. In order to explain further the differential effects of salts on protein stabilization, experiments over a wide concentration range are necessary to discriminate the "salting out" effect from the preferential salt binding (salting in) effects. The $MgCl_2$-induced refolding of HPC is accompanied by induction of significant secondary and tertiary structures. At low pH, there is net positive charge on the protein; so, it is more likely that it is the anions that are the key components in the action of salts. One indication that the effect involves a significant direct ion interaction with the protein is that although both sulfate and chloride have stabilizing effects on the protein, sulfate is a known kosmotropic (stabilizing) agent for protein, but chloride is neutral in this regard [45]. 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 presence of salts. Thus, chloride ions at higher concentration (3 M) bind to the positively charged HPC present at pH 2.0 and lead to stability of 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 [46]. All these effects taken together explain differential effects of salts in protein stabilization.

The overall results suggest that the acid-induced state of HPC in the presence of polyols and salts refolds into a conformation that lies in between the native and acid-denatured form. Among polyols, glycerol showed more protein stabilizing effect as compared to sorbitol. Figures 3 and 4 reveal native-like secondary structural features of HPC at pH 2.0 in the presence of glycerol as shown by the far UV-CD, increased binding of ANS compared to the native state although less than that of the acid-denatured state, blue shift of intrinsic fluorescence measurement indicating nonpolar environment of aromatic amino acid residues, but has slightly increased ellipticity as compared to the acid-induced state in the near UV region as shown in Fig. 7. Thus, with these features the glycerol-induced state is different from the acid-induced state present at pH 2.0. On the basis of the above considerations, it can be regarded as a state in between the native and acid induced state at pH 2.0. The decreased binding of ANS observed in this glycerol-induced state can be explained as unfolding of ANS binding sites or an increased ordering of tertiary structure that makes ANS binding sites in the protein more inaccessible. ANS binds to hydrophobic patches on protein [47]. Although in the native state ANS binding sites are buried deep in the protein interior, increased accessibility of ANS to these sites in the intermediate states results from the loose packing of residues in this form [48]. Since both intrinsic fluorescence and near UV-CD show the glycerol-induced state resembling the native state, it suggests ordering of tertiary structure. The case with the $MgCl_2$ state is similar, which has conformation in between the native and acid-induced states as can be seen in Figs. 5c, 6, and 7. Thus on the basis of these studies it can be concluded that both polyol and salt-induced states are different from the acid-induced states at pH 2.0. As the degree of preferential exclusion of a cosolvent is directly proportional to the protein surface area, the system under a particular set of conditions favors the protein state with the smallest area [49] and preferential exclusion of cosolvents leads to increased compactness of protein compared to the acid-induced state, especially in view of the unfavorable interactions between water and the polypeptide backbone [50]. This explains the midway conformation lying in between the native and acid-induced state at pH 2.0 of the cosolvent-induced states of HPC. Cystatins have an important role to play as thiol protease inhibitors in normal body processes owing to their cysteine proteinase inhibitory activity. Thus, the above observations shed some light on the possible structural alterations in HPC, which may in turn effect its normal functioning.

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