Disorder–order transition of λ CII promoted by low concentrations of guanidine hydrochloride suggests a stable core and a flexible C-terminus

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The CII protein of bacteriophage λ, which activates the synthesis of the λ repressor, plays a key role in the lysis–lysogeny switch. CII has a small *in vivo* half-life due to its proteolytic susceptibility, and this instability is a key component for its regulatory role. The structural basis of this instability is not known. While studying guanidine hydrochloride-assisted unfolding of CII, we found that low concentrations of the chaotrope (50–500 mM) have a considerable effect on the structure of this protein. This effect is manifest in an increase in molar ellipticity, an enhancement of intrinsic tryptophan fluorescence intensity and a reduction in ANS binding. At low concentrations of guanidine hydrochloride CII is stabilized, as reflected in a significant decrease in the rate of proteolysis by trypsin and resistance to thermal aggregation, while the tetrameric nature of the protein is retained. Thus low concentrations of guanidine hydrochloride promote a more structured conformation of the CII protein. On the basis of these observations, a model has been proposed for the structure of CII wherein the protein equilibrates between a compact form and a proteolytically accessible form, in which the C-terminal region assumes different structures.

Keywords: bacteriophage λ; HflB protein; proteolysis; genetic switch; lysogeny.

The transcriptional activator protein CII is a key element in the decision that governs the switching of λ phage development into one of its two alternate pathways, namely lytic or lysogenic [1,2]. A small protein of 97 amino acids, CII exists as a tetramer in the native state [3]. It is a relatively unstable protein, with a very small half-life *in vivo* [4] owing to its proteolytic degradation by the host protease HflB [5,6]. This instability of CII is essential for the λ decision [7]. Under conditions where CII is stabilized, it activates the transcription of the λ repressor (*cI*) and integrase (*int*) genes, promoting lysogeny; while the degradation of CII causes the phage to continue its development in the lytic pathway [1,2]. CII thus serves as an excellent model to study regulation of gene expression modulated by proteolysis. Such stability-mediated control exists in various systems, both in eukaryotes and prokaryotes, as has been reported for p53, RpoS, σ[32], etc. [8–10].

CII has been studied in great detail in terms of its transcription activation [11], interaction with RNA polymerase [12,13], or HflB-mediated proteolysis [4,6]. However, the structural and/or thermodynamic basis for the instabi-

lity of this protein had not been explored to date, except for a recent report involving CII truncated at the C-terminus [14]. In the present study, unfolding experiments were undertaken for CII. These provide deeper insights into the folding pathway as well as into the thermodynamic stability of folded native proteins. Denaturation of proteins using high concentrations of chaotropic chemicals, e.g. guanidine hydrochloride (GdnHCl) or urea, is a well known technique that has been used to study the unfolding of proteins. Such chemically induced denaturation of proteins results in a gradual loss of their secondary and tertiary structures. While carrying out GdnHCl-assisted equilibrium unfolding experiments with CII, we observed that low concentrations of GdnHCl (below 1 M) do not lead to unfolding of the protein. On the contrary, these conditions apparently promote a more ordered structure. We have carried out a systematic study of this interesting phenomenon, the results of which are presented in this communication.

Materials and methods

Materials

The CII protein was over-expressed in *Escherichia coli* BL21(DE3) [15] strain harboring the recombinant plasmid pAB305 [16], containing the *cII* gene downstream of T7 promoter. The recombinant protein was purified to 99% homogeneity using the purification protocol already reported [16]. The protein concentration was determined spectrophotometrically using $\varepsilon_{280} = 7.2 \times 10^4$ M^{-1}·cm^{-1} for the tetramer [3]. GdnHCl and Tris base (Ultrapure grade) were purchased from Life Technologies (Maryland, USA).

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Trypsin was from Sigma (St. Louis, MO, USA). All other chemicals, obtained locally from Merck (India), were of analytical grade.

For all experiments, the protein concentration is reported as the concentration of monomeric CII. Unless otherwise stated, experiments were carried out at room temperature (25 ± 2 °C) in 20 mM Tris/HCl, pH 8.0, containing 1 mM EDTA and 100 mM NaCl (buffer E).

Circular dichroism and fluorescence experiments

Far-UV CD and fluorescence data were recorded using 9 μM protein in buffer E containing GdnHCl or other salts to the desired concentration as and when required. Near-UV CD spectra were recorded using 2 μM protein solution in the same buffer. A JASCO J600 spectropolarimeter was used to record the CD spectra. For far- and near-UV CD, cuvettes of 0.1- and 10-cm pathlengths were used, respectively.

Intrinsic tryptophan fluorescence ($\lambda_{ex} = 295$ nm, $\lambda_{em} = 342$ nm) and ANS fluorescence ($\lambda_{ex} = 360$ nm, $\lambda_{em} = 495$ nm) were measured using a Hitachi F-3000 spectro-fluorimeter with 5 nm bandpass for both excitation and emission. For ANS binding experiments, ANS was used at a final concentration of 15 μM. Fluorescence lifetime measurements were carried out using a time-resolved spectro-fluorimeter assembled using components from Edinburgh Analytical Instruments, UK.

Thermal aggregation

Thermal aggregation of CII (12 μM in buffer E) was monitored using light scattering at 360 nm in a spectroflu-orimeter (Hitachi F-3000). Wavelength and bandpass were set at 360 and 2.5 nm, respectively, for both excitation and emission. The protein sample within the sample chamber was heated to the desired temperature using a waterbath temperature controller (NesLab Inc) and allowed to equili-brate for 3 min before monitoring the scattering. The temperature of the protein sample was measured using a digital thermometer (Hanna Instruments).

Analytical gel-filtration experiments

Analytical gel-filtration experiments were performed in an ÄKTA® FPLC system (Amersham Pharmacia Biotech) using a Superdex 75 HR 10/30 column. 50 μg of the protein was injected at a time. The column was pre-equilibrated with the elution buffer (buffer E) containing GdnHCl as and when mentioned.

One-dimensional FT-NMR experiments

FT-NMR experiments were carried out using a Bruker DRX-500 NMR machine at 300 K. The protein (500 μM) was taken in 20 mM sodium phosphate buffer, pH 7.5, containing 200 mM NaCl. GdnHCl was added to the buffer to the required concentration when mentioned. NMR spectra were recorded in 90% H_2O/10% D_2O (v/v). Water suppression was achieved using WATERGATE pulse sequence [17]. Diffusion measurements were made using stimulated echo-based pulse sequence as described [18], with a typical diffusion delay of 500 ms.

Partial proteolysis

Partial proteolysis of CII (10 μg) was carried out at room temperature (25 ± 2 °C) in a 20-μL reaction volume. The substrate (CII) to enzyme (trypsin) ratio was maintained at 100 : 1 (w/w). Proteolysis was limited to the desired time of incubation by subsequent addition of phenylmethanesulfo-nyl fluoride (to a final concentration of 5 mM) and SDS/PAGE sample buffer. The samples were analyzed in a 10–20% (w/v) acrylamide gradient SDS/PAGE followed by Coomassie blue staining.

Results

CD spectroscopy

CD measurements, both at far- and near-UV regions, provide excellent means of studying structural and con-formational features of proteins. The effect of low concen-trations of GdnHCl on the secondary structure of CII was examined by measuring the CD at 220 nm as a function of GdnHCl concentration. A significant increase (≈ 30%) in the molar ellipticity was observed (Fig. 1A), which leveled off around 0.2 M GdnHCl. The CD remained unchanged at this elevated level up to 0.7 M GdnHCl, beyond which the CD started decreasing and characteristic unfolding transi-tions for the protein were observed (data not shown). This increase of CD is also evident in the spectra presented in Fig. 1B, where the spectrum in the presence of GdnHCl (0.1 M) shows higher molar ellipticity values compared with that in the absence of GdnHCl.

In contrast, little change in the CD spectrum was observed in the near-UV region (Fig. 2) that is characteristic of aromatic residues. Therefore it is apparent that below 1 M, GdnHCl caused a conformational change in the CII protein with little change in tertiary interactions of the aromatic residues, while there was a significant gain of secondary structure. There are three tryptophan and two phenylalanine residues in the CII protein and tyrosine is absent. Interestingly, all the three tryptophans as well as one of the phenylalanines are located in the middle of the 97-residue peptide chain, between residues 40 and 60. Thus, a possible interpretation of the CD result is that there exists a core structure in CII, which is unaffected by low amounts of GdnHCl, while the flexible N- and/or C-terminus is affected.

Fluorescence spectroscopy

To further explore this conformational change, intrinsic tryptophan fluorescence of CII was examined in terms of intensity as well as the emission λ_{max} as a function of GdnHCl concentrations. Figure 3 shows the change in the intrinsic fluorescence intensity measured at the λ_{max} for the native protein (342 nm), as well as the change of emission maxima of CII, with increasing GdnHCl concentrations. It is evident that the emission maxima remained almost unchanged with the addition of GdnHCl up to 1 M, whereas the intensity increased marginally by about 6%. It is possible that this overall marginal effect may be a result of compensating changes in the spectroscopic characteristics of the three tryptophan residues. Time-resolved fluorescence
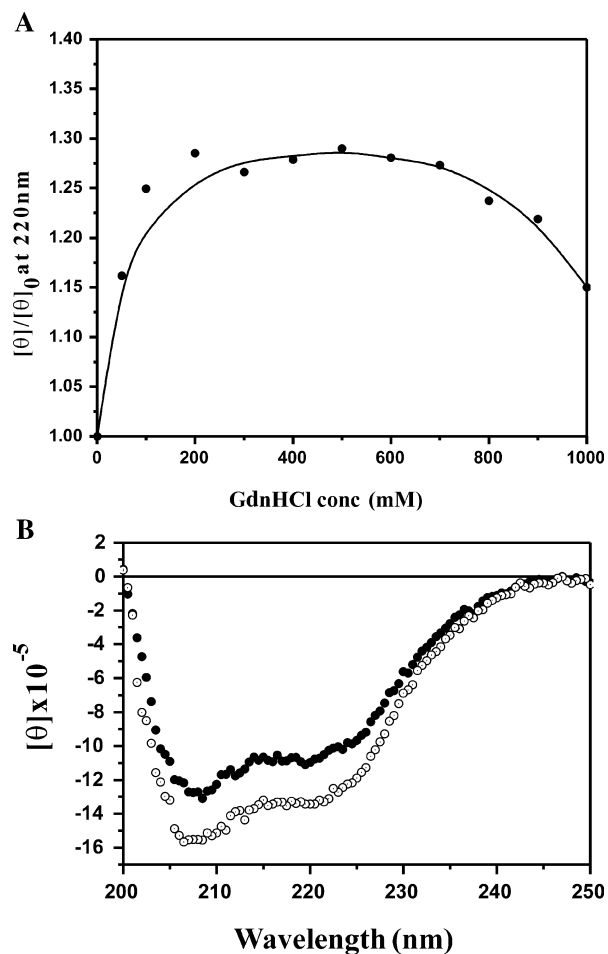
Fig. 1. Effect of the addition of GdnHCl (below 1 M) on the CII protein. (A) Relative molar ellipticity ($[\theta]/[\theta]_0$; $[\theta]_0$ represents molar ellipticity without GdnHCl) at 220 nm as a function of GdnHCl concentration (the curve represents a simple polynomial drawn through the points). (B) CD spectra of CII in the absence (●) and in the presence of 0.1 M GdnHCl (○) at the far-UV region. Nine micromoles of protein solution in buffer E was used.

Fig. 2. Near-UV CD spectrum of CII in the absence (●) and in the presence of 0.1 M GdnHCl (○). The data was recorded using a 10-cm pathlength cuvette with 2 μM protein solution in buffer E with or without 0.1 M GdnHCl.

Fig. 3. Change of intrinsic tryptophan fluorescence intensity (●) and emission maxima (○) for CII with GdnHCl concentration. The protein was incubated with varying amounts of GdnHCl in buffer E for 30 min. Fluorescence was recorded at room temperature in a cuvette of pathlength 1 cm ($\lambda_{ex} = 295$ nm, bandpass = 5 nm both for excitation and emission).

measurement of the CII protein was carried out both in the presence and absence of 0.1 M GdnHCl to investigate whether such a compensatory change occurs. The results, presented in Table 1, show that all the three tryptophan residues undergo small changes leading to an increase in the lifetime values for each. These results thus provide further evidence of the unchanged tryptophan environment upon addition of GdnHCl, supporting our conclusion drawn from the CD results.

ANS fluorescence

The detergent ANS (8-anilino-1-napthalenesulfonate) interacts with exposed hydrophobic surfaces of proteins. This binding can be monitored using the change of fluorescence intensity of the former. As ANS fluorescence does not change with the addition of GdnHCl (checked by control experiments, data not shown), the effect of low concentrations of GdnHCl on CII was also monitored using the
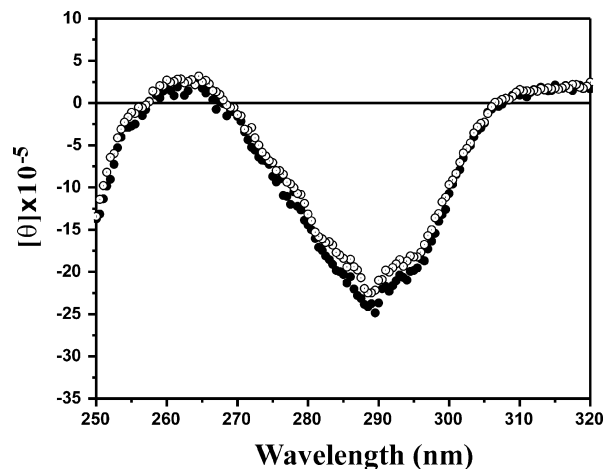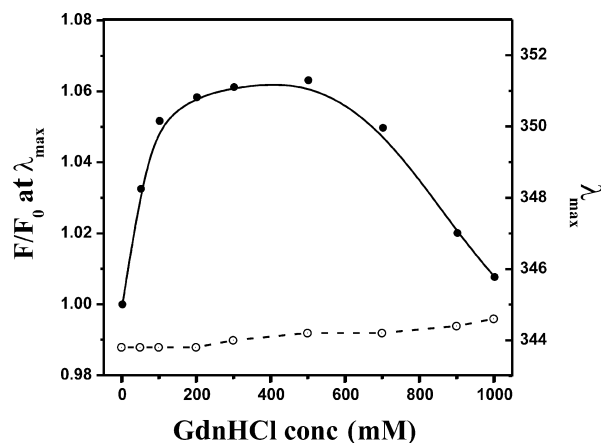
change in the fluorescence intensity of ANS as a probe. Figure 4 shows that the fluorescence intensity of ANS decreases with increase in GdnHCl concentration up to 0.75 M, and then gradually starts increasing with further increase of GdnHCl, probably as the protein unfolds exposing buried hydrophobic surfaces. These changes in the ANS fluorescence also point towards a structural change of CII leading to a reduction of exposed hydrophobic surface area on the protein molecule, below 1 M GdnHCl.

Effect of other salts

Salts are known to cause stabilization of proteins [19–21]. The apparent stabilization of CII observed as above could

Table 1. Fluorescence lifetime of three tryptophan residues in CII. Lifetime measurements were carried out in a time-resolved spectro-fluorimeter assembled using components from Edinburgh Analytical Instruments. The excitation was carried out at 297 nm using an N_2 flash-lamp and the emission was recorded at 340 nm for a total of 20 000 counts. The data showed a better fit to a triple exponential than to a double exponential, as judged by the significantly lower χ^2 values.

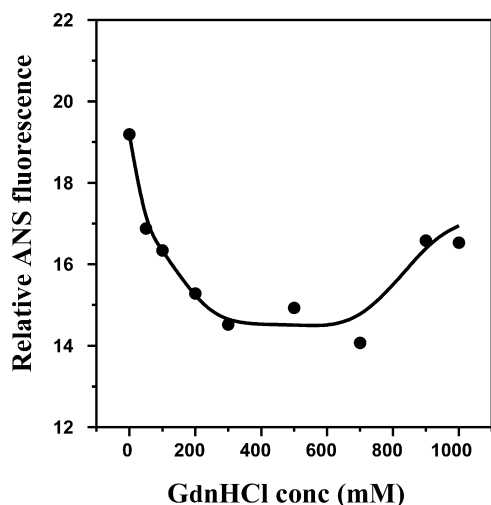| Residue (Trp)	Native		With 0.2 M GdnHCl	
	Lifetime (ns)	Amplitude (%)	Lifetime (ns)	Amplitude (%)
1	0.644	22.78	0.832	22.78
2	3.168	63.50	3.338	61.97
3	7.128	13.72	7.644	12.69

Fig. 4. Change of ANS fluorescence with increasing GdnHCl. ANS was added to the protein solution (incubated in presence of GdnHCl for 30 min) to a final concentration of 15 μM, and the fluorescence emission measured at 495 nm. Excitation wavelength was set at 360 nm.

thus either be a salt effect or be caused specifically by GdnHCl. To find out whether this was so, we carried out CD and ANS fluorescence measurements on CII in presence of other salts (sodium chloride, potassium phosphate at pH 8.0, and ammonium sulfate) and another nonionic chaotropic agent, urea. The results suggest that other salts can also cause stabilization of CII, albeit to a lower extent compared with that caused by GdnHCl (Fig. 5). The stabilizing effect follows the Hofmeister series of ion [22]. Urea, on the other hand, has negligible effect.

Thus it is clear that CII can undergo stabilization upon addition of salts in general. However, this stabilizing effect is most prominent in the case of the guanidium cation.

Temperature-induced aggregation

The CII protein undergoes irreversible thermal aggregation. Figure 6 shows that the aggregation begins at around 38 °C and is practically complete by 55 °C. In the presence of 0.2 M GdnHCl, however, the aggregation pattern was
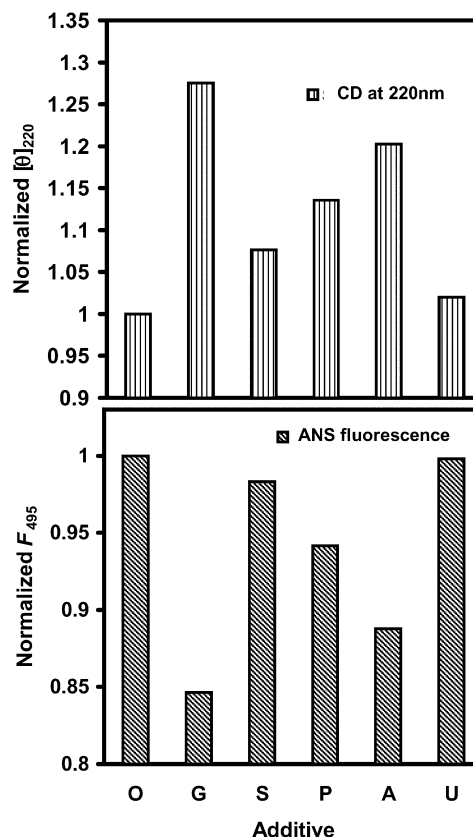

Fig. 5. Effect of salt on CII. Molar ellipticity (at 220 nm) and ANS fluorescence (λ_{ex} = 360 nm; λ_{em} = 495 nm) of CII protein (9 μM) in presence of GdnHCl, other salts and urea. Values shown were normalized by dividing by the corresponding ellipticity or fluorescence value for CII alone (without additive). All the additives were at a final concentration of 200 mM. 0, without any additive; G, with GdnHCl; S, NaCl; P, potassium phosphate, pH 8.0; A, ammonium sulfate; and U, urea.
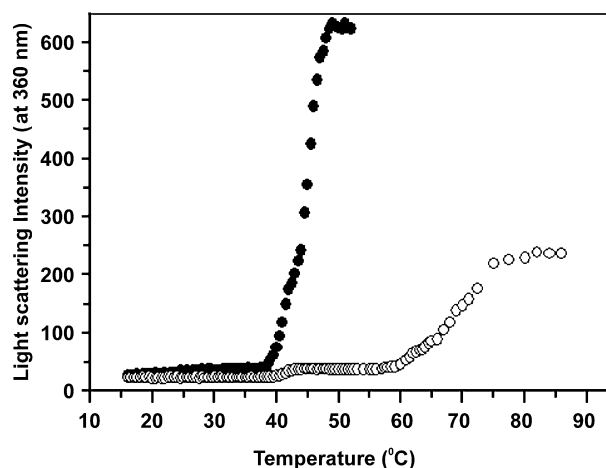

Fig. 6. Temperature-induced irreversible aggregation of CII in the presence (○) and in absence (●) of GdnHCl (0.2 M). The protein (in buffer E) was heated to a constant temperature and aggregation was monitored using static light scattering in a spectrofluorimeter using a 2.5 nm bandpass. Both excitation and emission wavelengths were set at 360 nm.

significantly different. It started at a much higher temperature (60 °C). Additionally, the extent of aggregation, as evident from the maximum relative light scattering, was reduced by more than 50%. This result is consistent with a structural compaction of the protein at low GdnHCl leading to a reduction in exposed hydrophobic surfaces.

Oligomeric status of the CII protein

Native CII exists as a homotetramer with a molecular weight of 4 × 12 kDa [3], but we wanted to know whether the protein was still tetrameric at low concentrations of GdnHCl. To investigate this, analytical gel filtration chromatography was carried out. Figure 7 shows a plot of the R_f value against the logarithm of molecular weight, obtained from gel filtration. The positions of CII alone and in the presence of 0.1 M GdnHCl are shown in this plot, along with protein molecular weight standards. Clearly, both these positions indicate tetrameric states of the protein.

The oligomeric status of the CII protein was further confirmed from diffusion coefficient measurements using NMR. The measured diffusion constants were $7.435 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ and $7.721 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ for CII alone and in presence of 0.2 M GdnHCl (viscosity uncorrected), respectively, which indicates that the Stokes' radius for CII remains practically unchanged at 0.2 M GdnHCl. These values also demonstrate the tetrameric organization of the protein when compared with corresponding values for

66.5 kDa bovine serum albumin ($6.1 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$) and 68 kDa hemoglobin ($6.9 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$) [23].

Interestingly, the small difference between protein organization, either with or without GdnHCl, is consistent with and points towards a compaction of the protein at low GdnHCl. This is reflected both in the reduction in the hydrated radius of CII (Fig. 7) and in the increased value of the diffusion constant upon addition of GdnHCl.

One-dimensional NMR experiments

The possible structural changes on CII at low GdnHCl concentration were also probed by 1D ^{1}H NMR experiments carried out with increasing concentrations of GdnHCl up to 0.5 M. The overall spectral pattern remained unchanged in the presence of GdnHCl, both in the down-field (amide) region (Fig. 8A) and the up-field region (Cα and sidechain, Fig. 8B). This indicates the absence of any global conformational change in the protein. It may be noted that there are several resonances with narrow line-widths in the spectrum for native CII (Fig. 8B) which move substantially upon addition of GdnHCl, and many of them undergo significant broadening. This suggests that some regions of CII under native conditions have shorter correlation times, i.e. greater mobility, than the overall tumbling motion. Broadening of these resonances upon addition of GdnHCl suggests a significant loss of this internal mobility.
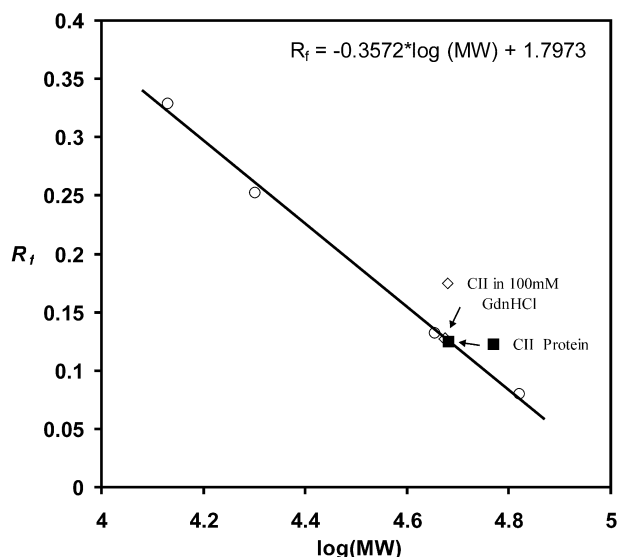

Fig. 7. Gel filtration analysis of the oligomeric status of the CII protein. The curve shows the plot of R_f value ($= \frac{V_e - V_0}{V_t - V_0}$; where V_e is the elution volume for a protein, V_0 the void volume and V_t the total column volume) for the four standard proteins (bovine serum albumin, ovalbumin, trypsin inhibitor and RNase A) against $\log_{10}$(molecular mass). The linear fit is also shown along with the equation. The positions of CII in presence and in absence of GdnHCl are indicated in the figure. The calculated molecular masses were 47 340.45 and 48 123.8 Da, respectively. Elution was carried out with buffer E for native CII and the standard proteins. 0.1 M GdnHCl was added to the same buffer for the elution of GdnHCl-treated CII.
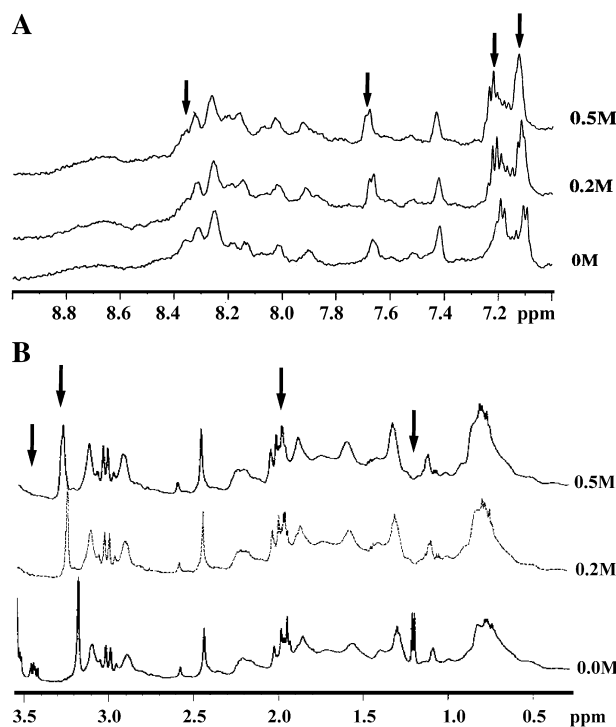

Fig. 8. One-dimensional NMR spectra of CII in the absence and presence of different concentrations of GdnHCl. (A) The NH region and (B) the Cα and the sidechain regions. Only minute changes are observed in the spectra with the addition of GdnHCl, as indicated by arrows. NMR spectra were recorded at 300 K in 90% H_2O/10% D_2O (v/v) using WATERGATE pulse sequence [17] for water suppression.

Partial proteolysis

Limited proteolysis is a powerful tool for studying protein conformation and their alterations [24,25]. Proteolytic enzymes preferably cleave at the flexible regions of an otherwise folded polypeptide chain. CD and NMR studies of the CII protein have indicated the presence of a rigid core and flexible region(s) within the molecule. These experiments also suggested that low concentrations of GdnHCl led to a stabilization of the flexible region(s). Such stabilization, which probably involves transition from a disordered to an ordered conformation, is likely to be reflected in the proteolytic digestion pattern and/or its kinetics. In view of the fact that the biological function of CII is modulated via its degradation by HflB, such studies also assume special importance for this protein.

When incubated with trypsin, CII is cleaved to a metastable 9 kDa polypeptide, CIIA, which is subsequently digested to a 7.4 kDa fragment, CIIB. Further incubation with the protease leads to a gradual waning of the 7.4 kDa polypeptide due to complete proteolysis. As seen in Fig. 9, both the intermediates are visible after 5 min of digestion at 25 °C. The larger polypeptide (CIIA) disappears by 10 min, while some CIIB is present even after 30 min of digestion. Substitution of trypsin by other proteases does not change the pattern of digestion (our unpublished results). When tryptic digestion was carried out in the presence of 0.1 M GdnHCl, no change in the proteolytic cleavage pattern was observed. However, the rate of digestion decreased significantly so that even after 30 min, some undigested protein could be seen, along with the CIIA and CIIB polypeptides. This decrease in the rate of proteolysis by trypsin can be attributed to an alteration of CII conformation as 0.1 M GdnHCl reduces trypsin activity by a meager 6% (as measured spectrophotometrically by the rate of degradation of BAEE, a nonpeptide substrate for measuring the catalytic activity of trypsin).

From the size of the proteolytic intermediates (9 and 7.4 kDa) it is clear that proteolysis occurs at the terminal region(s) of the polypeptide chain, thereby supporting the proposition that either one or both termini of the protein is quite flexible and remains in a disordered state that is readily accessible to proteolytic enzymes. The unaltered pattern of proteolysis also indicates that the overall conformation of

Fig. 9. Partial proteolysis of CII protein with trypsin. Ten micrograms of CII in 20 μL reaction buffer (buffer E) was incubated with trypsin (protein/trypsin = 100 : 1) for the indicated time intervals either alone (−) or in the presence of 0.1 M GdnHCl (+). Reactions were stopped by the addition of SDS/PAGE loading buffer and analyzed on a gradient SDS/PAGE (10–20%) with Coomassie brilliant blue staining. (Lanes: MW, molecular mass marker; lane 0′, undigested CII; all other lanes indicate the time of incubation in minutes).

CII remains unchanged at 0.1 M GdnHCl. At this concentration, GdnHCl appears to stabilize the flexible terminal region(s) of the protein leading to the reduction of the rate of proteolysis.

Discussion

At low concentrations, GdnHCl is found to have a stabilizing effect on the CII protein, which is reflected in (a) enhancement of far-UV molar ellipticity; (b) reduction in ANS binding; (c) reduction in the rate of proteolysis; and (d) reduced thermal aggregation. This stabilization probably occurs because GdnHCl at low concentrations shifts the equilibrium towards a more ordered state of the protein, which is in equilibrium with a partially disordered structure. The pattern of proteolysis of CII in the presence of GdnHCl is remarkably similar to that in its absence, except that the rate slows down to a great extent. This is consistent with two rapidly equilibrating conformations in which proteolysis occurs from the more disordered state. This partial disorder under native conditions is also supported by NMR data. The nature of the disordered state is difficult to establish in detail. However, unaltered near-UV CD, tryptophan fluorescence emission maxima and fluorescence lifetimes of the disorder–order transition suggest that the central portion of the protein, where most of the aromatic residues are situated, is relatively unaffected. The observed proteolysis pattern also suggests that digestion occurs at either one or both of the terminal ends. The presence of a large flexible region at the C-terminus was indicated by NMR studies (our unpublished data). The C-terminus has recently been shown to be a target for rapid proteolysis [14]. Thus it appears that CII protein consists of a stable core in the middle and a flexible C-terminal region. However the present data do not rule out a possible N-terminal flexible region also. As the stability of CII is of utmost importance for its biological function, it is possible that disordered regions in the protein serve as the initial attacking point for proteases and thereby play a key regulatory role. The protease that plays a crucial role in the degradation of CII *in vivo* is HflB. The homology model of HflB [26,27] suggests it to be a donut-shaped hexamer with its active site located in the central cavity. Such a structure provides a logical explanation of the fact that HflB specifically cleaves denatured polypeptides [28], as only denatured peptides would be able to enter the cavity. Clearly, terminal disordered regions are good candidates for proteolysis by this protease. Diffusion coefficient measurements (using NMR) and analytical gel filtration carried out to verify the oligomeric status of this stable conformation have shown that the protein retains its tetrameric status, suggesting that the flexible terminal region is not involved in tetramerization of the protein.

The above features of CII may be represented by a schematic model (Fig. 10) that shows the two alternative conformations of the protein in equilibrium with each other.

Why low concentrations of GdnHCl act as the most effective ionic agent that can promote the disorder–order transition in this protein is not very clear at present. It is possible that in addition to general structure-promoting effects, GdnHCl binds to some pockets in the ordered
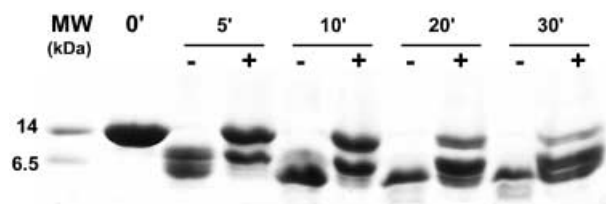
mvrankrnea lriesallnk iamlgtekta eavgvdksqi srwkrdwipk
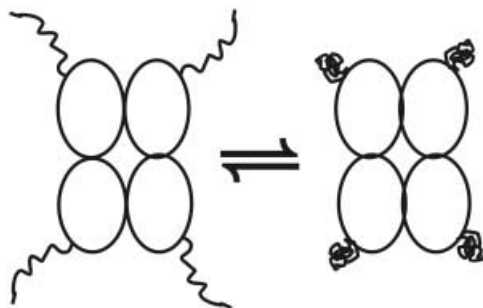fsmllavlew gvvdddmarl arqvaailtn kkrpaaters eqiqmef

Fig. 10. Schematic model depicting the two equilibrating conformations of CII. In the proteolytically accessible 'open' conformation (left), terminal flexible region(s) exist in an extended state, while in the 'closed' form (right) the protein assumes a more compact conformation. Both the conformations are tetrameric, and have the folded core region. GdnHCl stabilizes the compact conformation thereby shifting the equilibrium towards the right. The complete amino acid sequence of CII is also shown with the tryptophan residues highlighted.

conformation of the protein, thus shifting the equilibrium. More detailed investigations on the interaction of the guanidium ion with this protein are needed to understand the physico-chemical basis of the order-promoting effect of low concentrations of GdnHCl.

Conclusion

We conclude that the CII protein of bacteriophage λ contains a stable core and a flexible, disordered C-terminus. This disordered region may be responsible for protease-mediated instability of CII, crucial for its regulatory function.

Acknowledgements

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