5. *Methanococcus jannaschii* RelBE toxin -antitoxin system

5.1. Results

5.1.1. Circular dichroism spectropolarimetry of *Methanococcus jannaschii* RelBE complex

The far-UV circular dichroism spectra of the 139 amino acid *Methanococcus jannaschii* RelBE complex in 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl, measured at 25 °C revealed minima at 207 and 222 nm (Figure 5.1). About 39% α-helix, 15% β-sheet and 46% random coil structures were calculated with the *K2d* algorithm (*173*), that was used because the presence of high salt concentration in buffer limits measurement of the spectra to 195 nm.

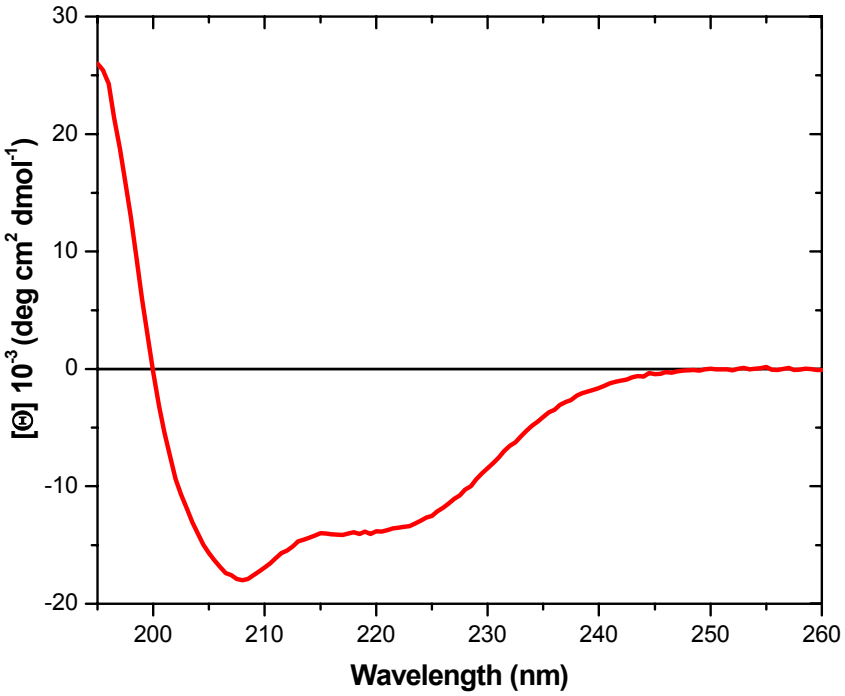

Figure 5.1: Far-UV CD spectra of *Methanococcus jannaschii* RelBE complex. Circular dichroism was measured at 25 °C at a concentration of 1.2 g/L in 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl.

5.1.2. Hydrodynamic properties and stoichiometry

Hydrodynamic properties and stoichiometry of *Methanococcus jannaschii* RelBE complex in 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl, were measured by gel filtration (size exclusion chromatography). On lowering sodium chloride concentration below 0.3 M in buffer, protein tends to precipitate.

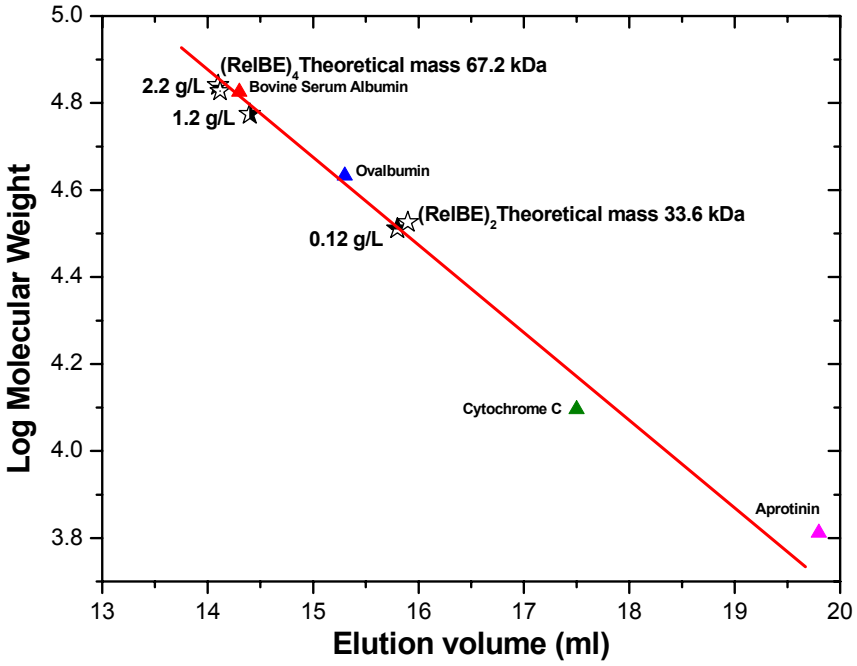

Figure 5.2: Molecular mass determination of *Methanococcus jannaschii* RelBE complex by size exclusion chromatography. Molecular standards for calibration were: Bovine serum albumin (67 kDa); Ovalbumin (43 kDa); Cytochrome C (12.5 kDa) and Aprotinin (6.5 kDa). The observed apparent molecular masses of RelBE are ~32.42 kDa, ~59.5 kDa and ~69.2 kDa for an input concentration of 0.12 g/L, 1.2 g/L and 2.2 g/L respectively in 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl, 25 °C. The theoretical mass of $(RelBE)_2$ and $(RelBE)_4$ are 33.6 kDa and 67.2 kDa respectively.

5.1.2.1. Size exclusion chromatography

Gel filtration experiments were performed at concentration of 0.12 g/L, 1.2 g/L and 2.2 g/L. Elution peak from 0.12 g/L has an asymmetric shape, with tails

to higher elution volumes, in contrast concentration 1.2 g/L and 2.2 g/L elution resulted in a single symmetric peak. The peak positions indicated apparent molecular masses of approximately 32.42 kDa, 59.5 kDa and 69.2 kDa for 0.12 g/L, 1.2 g/L and 2.2 g/L respectively (Figure 5.2). The observed molecular masses reveal very high concentration dependence. Theoretically, RelBE complex, $(RelBE)_2$ and $(RelBE)_4$ have molecular masses of 16.8 kDa, 33.6 kDa and 67.2 kDa, respectively.

5.1.3. Denaturant-induced unfolding

Denaturant unfolding of *Methanococcus jannaschii* RelBE complex was analyzed by monitoring changes in the far ultraviolet circular dichroism (changes predominantly in secondary structure). As the CD spectra of the RelBE complex revealed greater negative ellipticity in the region of 208-228 nm, a region with bands characteristic of α-helix and β turns, respectively; therefore; unfolding of RelBE complex by Gdn-HCl was monitored measuring changes of the CD ellipticity at 222 nm (Figure 5.3).

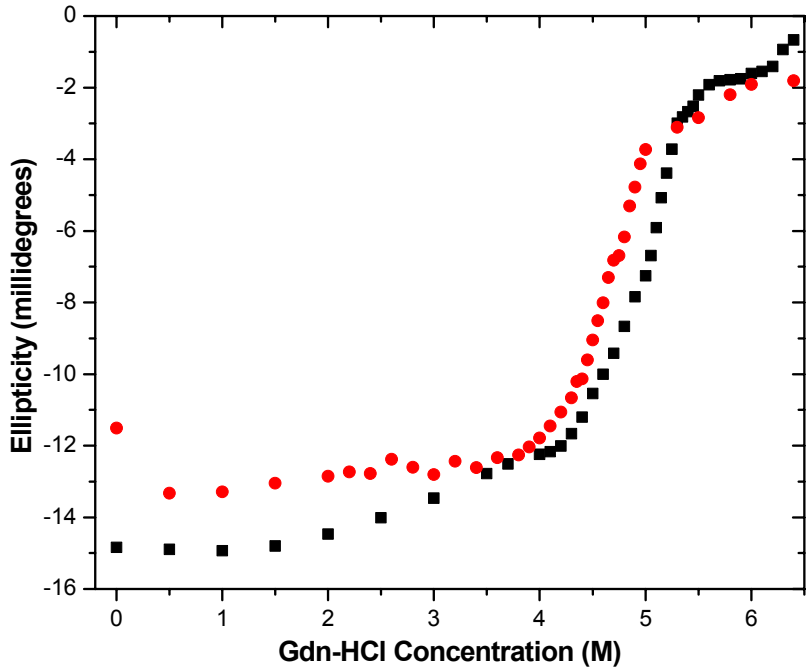

Figure 5.3: Gdn-HCl induced unfolding of *Methanococcus jannaschii* RelBE complex as monitored by circular dichroism. Gdn-HCl induced unfolding of 0.28 g/L and 1.8 g/L RelBE complex are represented by red circles and black squares, respectively. 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl containing Gdn-HCl concentrations were used for unfolding as indicated. CD ellipticity changes were measured at 222 nm and 20 °C. C½ values for half unfolding denaturant concentration was calculated as 4.6 and 5 M for 0.28 g/L and 1.8 g/L RelBE complex respectively.

5.1.3.1. RelBE Gdn-HCl unfolding as monitored by circular dichroism

Denaturation curves of RelBE complex were measured at concentration 0.28 g/L and 1.8 g/L (Figure 5.3). A marginal lower stability was observed for the lower protein concentration. $C_{½}$ values for half unfolding denaturant concentration was calculated as 4.6 and 5 M for 0.28 g/L and 1.8 g/L RelBE complex, respectively. A concentration dependent complex unfolding mechanism can be observed from the obtained curves; however increase in protein concentration revealed a marginal stability difference pointing to higher denaturant stability with higher protein concentration. Therefore concentration

dependence is evident. Dissociation steps or intermediates in multi-component system could not be seen. Irrespective of measured concentration, the RelBE complex unfolding was found to be closely associated with the complex dissociation to its individual components.

5.1.4. Temperature-induced unfolding

5.1.4.1. Circular dichroism

5.1.4.1.1. Native buffer conditions

Thermal stability of *Methanococcus jannaschii* RelBE complex at 0.12 g/L concentration in 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl was monitored by circular dichroism. Spectra were measured from 5 to 95 °C with steps of 10 °C (Figure 5.4).

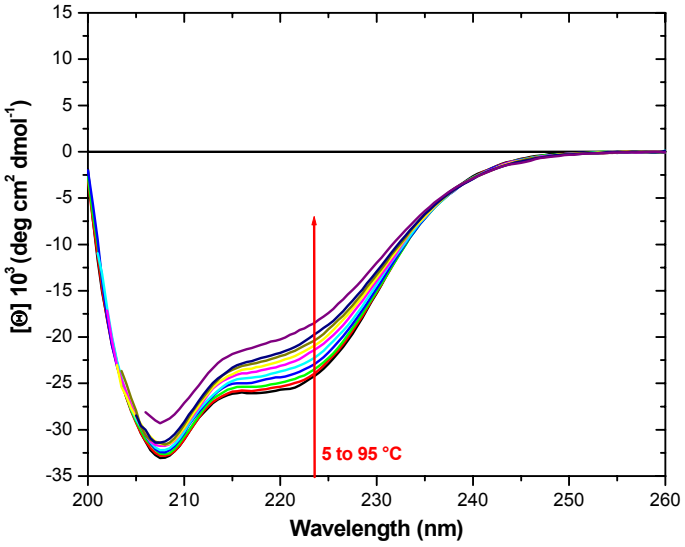

Figure 5.4: Thermal unfolding spectra of *Methanococcus jannaschii* RelBE complex as measured by circular dichroism. CD spectra's were measured with 0.12 g/L *Methanococcus jannaschii* RelBE complex in 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl. Upward red arrow indicates the spectral shift with increase of temperature from 5 to 95 °C.

Spectral changes were monitored at 222 nm are shown in Figure 5.5. Linear loss of negative ellipticity from ~-26 · 10^3 to ~-20.5 · 10^3 deg·cm^2·dmol^{-1} was observed. It is difficult to determine half transition temperatures from the CD melting curve as loss of secondary structure or unfolding was not completed in the measured temperature range.

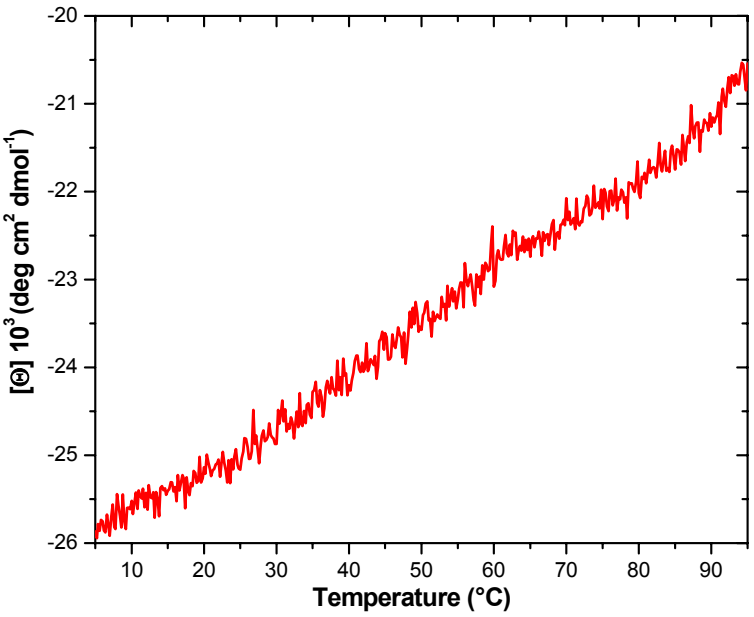

Figure 5.5: Thermal unfolding curve of *Methanococcus jannaschii* RelBE complex as monitored by change in ellipticity at selective wavelength of 222 nm by CD. CD ellipticity was measured with 0.12 g/L *Methanococcus jannaschii* RelBE complex in 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl. The shift in molar ellipticity at 222 nm is plotted against increase in temperature.

Reversibility of thermal melting was checked by slow cooling down of the samples back to 15 °C from 95 °C. Near 95 % reversibility was observed at measured concentration (Figure 5.6). Even at 95 °C, loss in negative ellipticity was marginal, suggesting significant amount of intact secondary structure at the temperature.

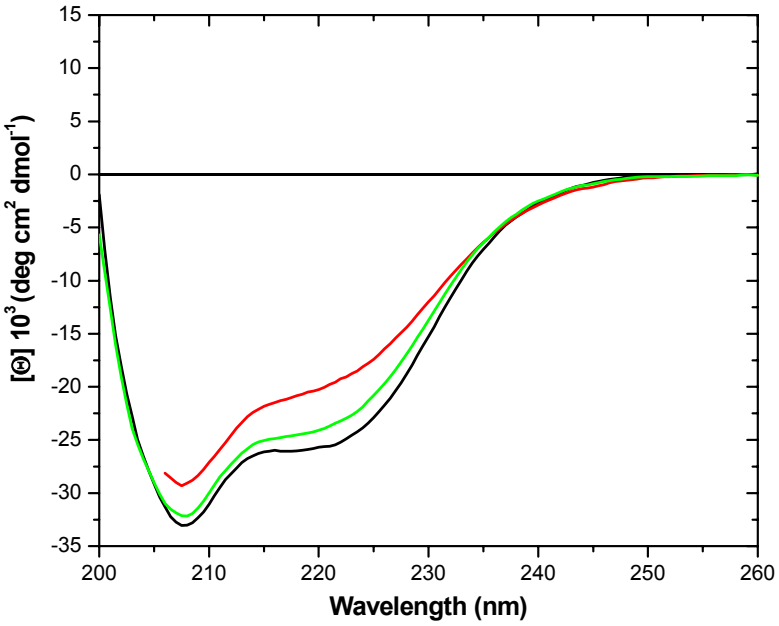

Figure 5.6: Thermal unfolding spectra of *Methanococcus jannaschii* RelBE complex (including renaturation spectra). CD spectra's were measured with 0.12 g/L *Methanococcus jannaschii* RelBE complex in 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl. Black, red and green lines indicate spectra's at 5, 95 and back to 15 °C (renaturation).

5.1.4.1.2. Denaturing buffer conditions

Thermal unfolding curves of *Methanococcus jannaschii* RelBE complex were monitored in buffer with 1.78 M, 2.78 M and 3.85 M Gdn-HCl. Protein in native like buffer conditions is highly stable and high temperature results in the formation of aggregates during unfolding. Therefore, Gdn-HCl was used to destabilize the interactions in the temperature range where CD signal at 222 nm in Gdn-HCl free buffer stays unchanged. CD ellipticity at 222 nm was measured with pathlength of 0.1 and 0.01 cm in the presence of 1.78 M, 2.78 M and 3.85 M Gdn-HCl + 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl. Measurements were performed at low concentration of 0.42, 0.5 and 0.46 g/L (Figure 5.7), and higher concentration of 1.74, 1.52 and 2.52 g/L (Figure 5.8). The shift in molar ellipticity at 222 nm is plotted against increase in temperature.

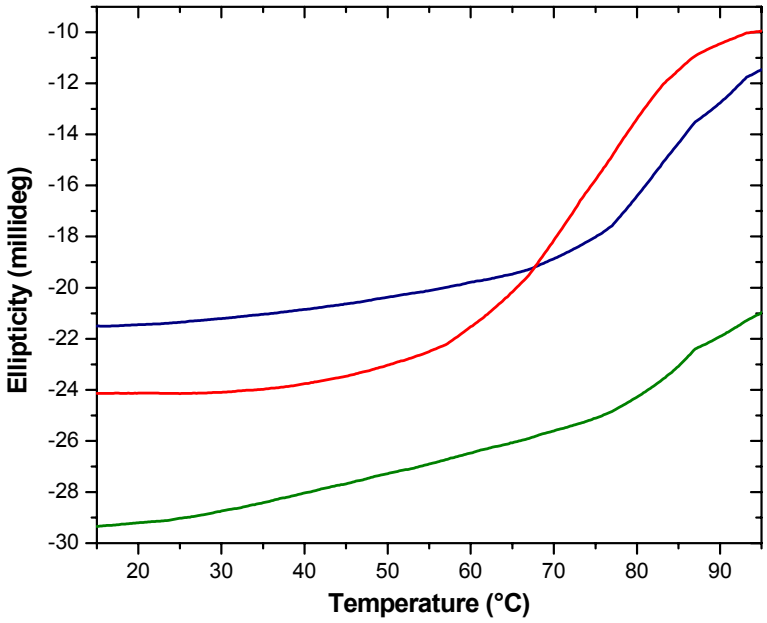

Figure 5.7: Smoothed thermal unfolding curves of *Methanococcus jannaschii* RelBE complex in the presence of Gdn-HCl as monitored by change in ellipticity at selective wavelength of 222 nm by CD. CD ellipticity was measured with pathlength of 0.1 cm in 1.78 M (green line), 2.78 M (blue line) and 3.85 M (red line) Gdn-HCl + 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl with 0.42, 0.5 and 0.46 g/L *Methanococcus jannaschii* RelBE complex respectively. The shift in molar ellipticity at 222 nm is plotted against increase in temperature. For comparative purpose all curves are factored to 0.5 g/L.

In Figure 5.7 and 5.8, curves reveal a change in negative ellipticity with increase of temperature to 95 °C. Transition region of the curves cannot be defined for 1.78 and 2.78 M Gdn-HCl, as the curves obtained are in the limited temperature range where unfolding is partial. At 3.85 M Gdn-HCl a sigmoidal unfolding curve was obtained with change in slope at about 55 °C and plateau values were reached at 90 °C. For 3.85 M Gdn-HCl, ellipticities clearly define a post transition region. A T_m value of 74.5 °C and 73.2 °C and ΔH value of 58.7 and 60.4 kcal/mol has been calculated for low and high protein concentration, respectively.

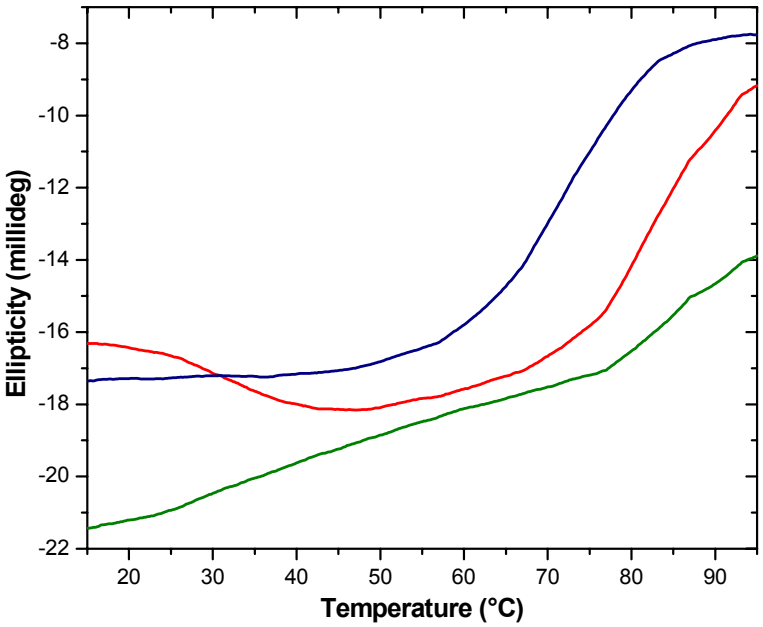

Figure 5.8: Smoothed thermal unfolding curves of *Methanococcus jannaschii* RelBE complex in the presence of Gdn-HCl as monitored by change in ellipticity at selective wavelength of 222 nm by CD. CD ellipticity was measured with pathlength of 0.01 cm in 2 M (green line), 2.78 M (red line) and 3.85 M (blue line) Gdn-HCl + 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl with 1.74, 1.52 and 2.52 g/L *Methanococcus jannaschii* RelBE complex respectively. The shift in molar ellipticity at 222 nm is plotted against increase in temperature. For comparative purpose curves were factored to 2.52 g/L.

5.1.4.2. Differential scanning calorimetry

5.1.4.2.1. *Native buffer conditions*

The thermal unfolding of 0.26 g/L of *Methanococcus jannaschii* RelBE complex in 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl has been examined by differential scanning calorimetry (Figure 5.9). Deconvolution of curve was not possible due to aggregation effects that started at about 95-100 °C clearly far below for completion of thermal unfolding.

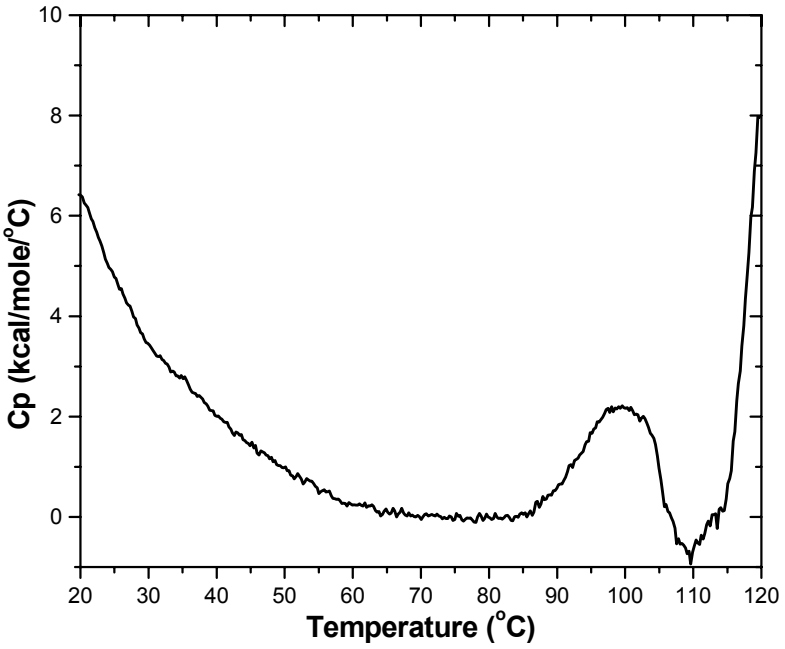

Figure 5.9: Differential scanning calorimetry of *Methanococcus jannaschii* RelBE complex. The experimental excessive heat capacity curve of 0.26 g/L of *Methanococcus jannaschii* RelBE complex in 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl is shown by the thick line.

5.1.4.2.2. *Denaturing buffer conditions*

To overcome the problem of not having well defined post-transition region, protein samples were destabilized by incubating protein samples with different concentrations of Gdn-HCl in buffer likely as for CD thermal melting.

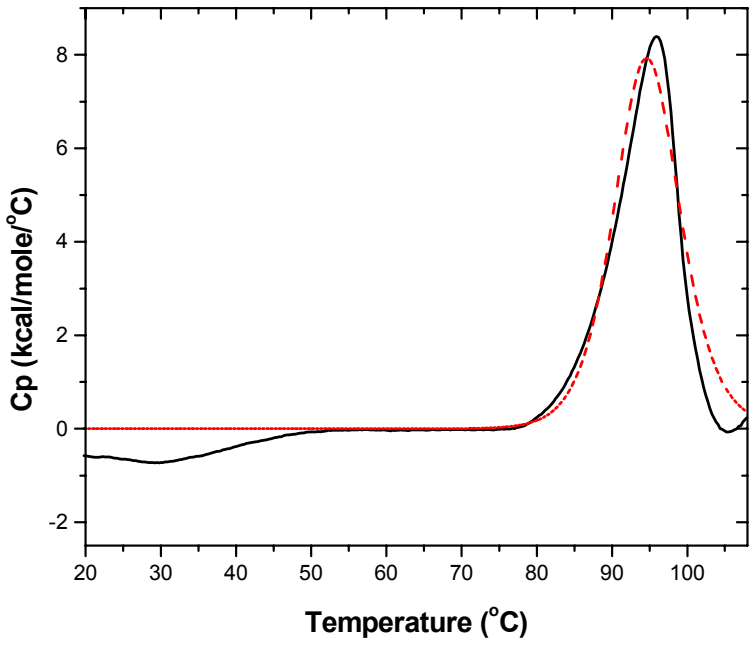

Figure 5.10: Differential scanning calorimetry of *Methanococcus jannaschii* RelBE complex in the presence of 1.78 M Gdn-HCl in buffer. The experimental excessive heat capacity curve of 1.74 g/L of *Methanococcus jannaschii* RelBE complex in 1.78 M Gdn-HCl + 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl is shown by the thick line, and the dashed red line indicates the result of deconvolution analysis assuming a two-state transition.

Excessive heat capacity curves of *Methanococcus jannaschii* RelBE complex were measured in the presence of 1.78, 2.78 and 3.85 M Gdn-HCl in 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl. The obtained curves were fitted assuming a two-state transition (Figure 5.10, 5.11, 5.12 and 5.13). The fitted curve for 1.78, 2.78 and 3.85 M Gdn-HCl in 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl yielded the T_m and ΔH value as summarized in Table 5.1.

Table 5.1: Thermodynamic parameters for the *Methanococcus jannaschii* RelBE complex obtained by the model analysis of CD and DSC thermograms.

Method	Gdn-HCl concentration (M)	Protein concentration (g/L)	Melting temperature (T_m) (°C)	Enthalpy (ΔH) (kcal/mol)
DSC	0[a]	-	115.9 ±0.16 [a]	110.3 ±0.3 [a]
DSC	1.78	1.74	94.7 ±0.08	92.3 ±0.7
DSC	2.78	1.52	86.3 ±0.15	85.5 ±1.15
DSC	3.85	2.5	75.03 ±0.24	75.7 ±1.54
CD	3.85	0.46	74.5 ±0.14	58.7 ±2.32
CD	3.85	2.5	73.2 ±0.07	60.4 ±1.76

[a] after linear extrapolation to 0 M Gdn-HCl concentration.

Melting temperatures (T_m) and enthalpies (ΔH) obtained at different Gdn-HCl concentrations were linearly extrapolated to 0 M Gdn-HCl (Figure 5.14). Extrapolation resulted in melting temperature (T_m) of 115.9 ±0.16 °C and enthalpy (ΔH^0) of 110.3 ±0.3 kcal/mol (Table 5.1). There were significant differences between calculated ΔH values from CD and DSC at 3.85 M Gdn-HCl, possibly because CD is the measure of secondary structrural changes whereas DSC measures total heat capacity changes. However, at the end DSC is a more reliable method for calculating thermodynamic parameters than CD.

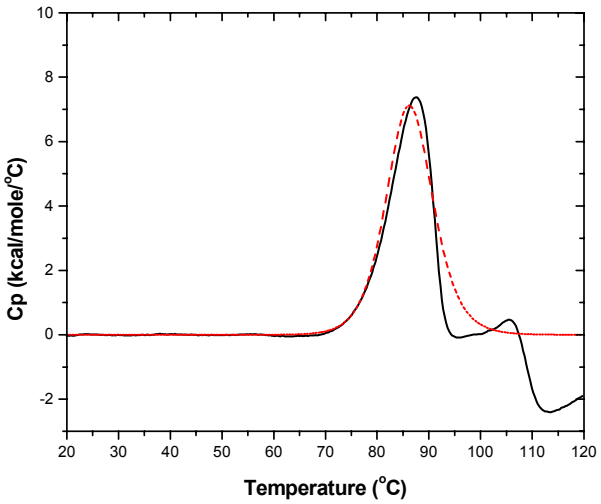

Figure 5.11: Differential scanning calorimetry of *Methanococcus jannaschii* RelBE complex in the presence of 2.78 M Gdn-HCl in buffer. The experimental excessive heat capacity curve of 1.52 g/L of *Methanococcus jannaschii* RelBE complex in 2.78 M Gdn-HCl + 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl is shown by the thick line, and the dashed red line indicates the result of deconvolution analysis assuming a two-state transition.

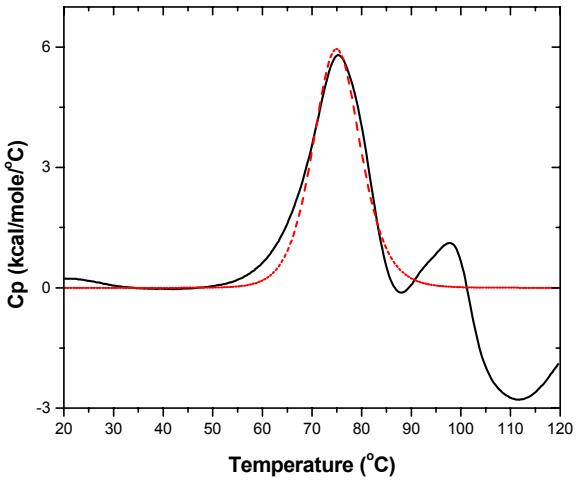

Figure 5.12: Differential scanning calorimetry of *Methanococcus jannaschii* RelBE complex in the presence of 3.85 M Gdn-HCl in buffer. The experimental excessive heat capacity curve of 2.5 g/L of *Methanococcus jannaschii* RelBE complex in 3.85 M Gdn-HCl + 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl is shown by the thick line, and the dashed red line indicates the result of deconvolution analysis assuming a two-state transition.

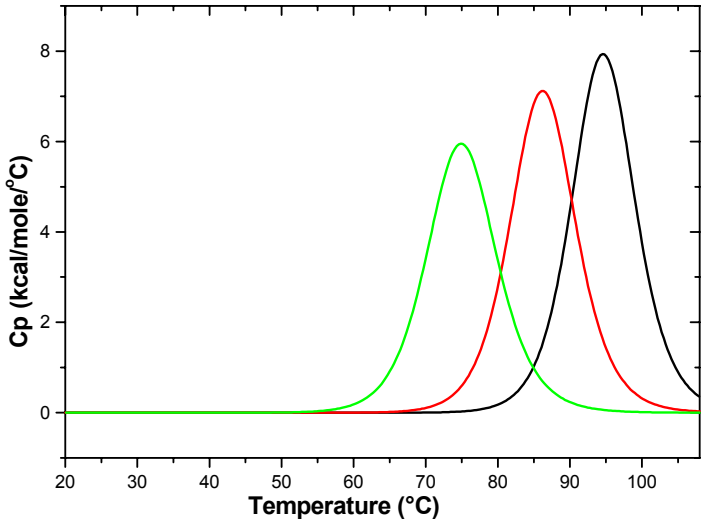

Figure 5.13: Deconvoluted excessive heat capacity curves of *Methanococcus jannaschii* RelBE complex in the presence of Gdn-HCl in buffer. The deconvulated excessive heat capacity curve of *Methanococcus jannaschii* RelBE complex in 1.78, 2.78 and 3.85 M Gdn-HCl in 20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl are shown by black, red and green line respectively. Deconvolution analysis was done assuming a two-state transition.

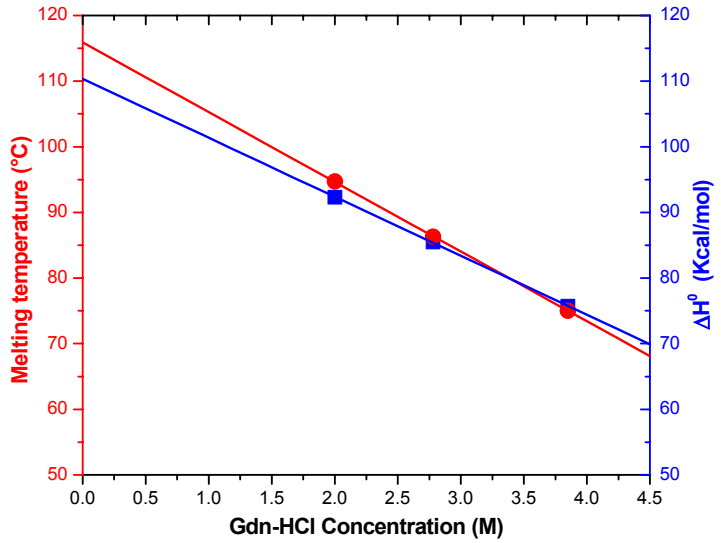

Figure 5.14: Plot of melting temperatures (T_m) and unfolding enthalpies (ΔH^0) of *Methanococcus jannaschii* RelBE complex *versus* Gdn-HCl concentrations, determined from differential scanning calorimetry measurements. T_m and ΔH^0 values are obtained by linear extrapolation of measured values to 0 M Gdn-HCl.

5.2. Discussion

Organisms that thrive at the perceived limits of temperature, pressure, pH, and salinity are classified as extremophiles, most of which belong to the class archaea. One of the interesting hyperthermophilic, methylotrophic, autotrophic and strict anaerobic organisms belongs to the class is *Methanococcus jannaschii* (Tax ID: 243232). It grows at pressure of up to more than 200 atm and over a temperature range of 48 to 94 degrees Celsius, with an optimum near 85 degree Celsius. Similar to *E. coli*, *Methanococcus jannaschii* also contains number of TA systems. Recent computational database mining revealed minimum 9 TA loci in the organism (*72*). Only one *i.e.* RelBE toxin -antitoxin system has attracted the attention of biologists up to now (*104*). However, another hyperthermophilic organism *Pyrococcus horikoshii* also contains similar RelBE system whose crystal structure has been already known (*108*). Interestingly, RelE homologues from Gram-positive bacteria and archaea inhibit translation in *E. coli* and cleave tmRNA. Although RelE proteins from bacteria and archaea are clearly homologous, their sequence similarities are modest (e.g. RelE from *E. coli* and *M. jannaschii* homologue share 18% identical and 40% similar amino acids only). The function of *relBE* loci in *M. jannaschii* is not known but, as in the case of *E. coli relBE*, may be related to regulation of translation during nutritional stress.

To gain more insight in RelBE toxin -antitoxin system behavior, we started studying physico-chemical properties of RelBE from *M. jannaschii*. The measured secondary structure revealed a dominating α-helical complex with modest amount of β-sheets (Figure 5.1). This observation is similar to the RelBE complex obtained from *E. coli*. Interestingly, RelBE from *M. jannaschii* exhibits a high stability towards heat and Gdn-HCl. Such exceptional stabilites have been observed for other proteins from hyperthermophilic organisms and explained by thermodynamic properties of their polypeptide chains. Oligomerization is one of the several mechanisms of thermal stabilization of a protein but no general

adaptive rules have been estabilished. In our FPLC experiments we observed a very high concentration dependence of the RelBE complex with change in protein concentrations varying from hetero-dimers to hetero-octamers (Figure 5.2).

Stability profile

Unfolding of complex in the presence of Gdn-HCl is a cooperative process with significant protein concentration dependence. Denaturant unfolding of RelBE complex was measured by changes in ellipticity revealed a high denaturant stability with $C_{½}$ 4.6 M and 5 M at concentrations of 0.28 g/L and 1.8 g/L, respectively (Figure 5.3). In multimeric two component proteins, intermediates are usually expected in transition profiles directing dissociation or differential stabilities of components. Unfortunately, we could not figure out any of the expected changes from our transition profiles. Our transition profiles revealed a very tight cooperative unfolding, concealing dissociation or individual unfolding phenomena. Therefore, unfolding phenomena is closely associated with dissociation of complex. Attempts to study heat denaturation of RelBE complex in physiological buffer conditions could also not resolve the unfolding into individual steps. We adopted circular dichroism and differential scanning calorimetry. Both techniques revealed a very high stability of complex towards heat. We measured excessive heat capacity curves of sample, but heat unfolding phenomena were found to be highly associated to the formation of aggregates. Therefore to attain a better understanding of the system stability we started combined usage of denaturant destabilization and thermal unfolding, which at the end resulted to approximate transition temperatures and unfolding energies.

RelBE complex found to be denaturant stable up to ~4 M Gdn-HCl therefore we started acquiring thermal transitions at 1.78 M Gdn-HCl concentration. At 1.78 M Gdn-HCl, minor destabilization of the complex against

thermal unfolding was observed by both CD and DSC. With CD, unfolding was partial as expected sigmoidal curves were not the case. We increased the destabilization by 3.75 M Gdn-HCl and followed unfolding by both CD and DSC. CD curves were fitted to two-state unfolding model and T_m and ΔH values have been calculated. From DSC, Obtained excessive heat capacity curves at 1.78, 2.78 and 3.85 M Gdn-HCl were successfully deconvoluted; considering symmetry of the curve which directs to two-state unfolding *i.e.* native and unfolded complex. T_m and ΔH values calculated from individual curves were linearly extrapolated to 0 M Gdn-HCl concentrations to obtain the T_m (116 °C) and ΔH (110.3 kcal/mol) value in the absence of denaturant (Table 5.1 and Figure 5.14). We found the maximal stability of RelBE complex around 115 °C which is no surprise for the organism growing in the optimal temperatures of 85 degree Celsius.