Folding and unfolding pathway of chaperonin GroEL monomer and elucidation of thermodynamic parameters

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The conformation and thermodynamic stability of monomeric GroEL were studied by CD and fluorescence spectroscopy. GroEL denaturation with urea and dilution in buffer leads to formation of a folded GroEL monomer. The monomeric nature of this protein was verified by size-exclusion chromatography and native PAGE. It has a well-defined secondary and tertiary structure, folding activity (prevention of aggregation) for substrate protein and is resistant to proteolysis. Being a properly folded and reversibly refoldable, monomeric GroEL is amenable for the study of thermodynamic stability by unfolding transition methods. We present the equilibrium unfolding of monomeric GroEL as studied by urea and heat mediated unfolding processes. The urea mediated unfolding shows two transitions and a single transition in the heat mediated unfolding process. In the case of thermal unfolding, some residual structure unfolds at a higher temperature (70–75 °C). The process of folding/unfolding is reversible in both cases. Analysis of folding/unfolding data provides a measure of ΔG


1. Introduction

In the cellular environment, newly synthesized polypeptides are at risk of aggregation, misfolding and degradation. The cell has a network of proteins which help in prevention of aggregation; promote efficient folding and intracellular transport. These proteins are categorized as Molecular Chaperones [1,2]. The GroEL protein encoded by the GroE gene of *Escherichia coli* was discovered as a critical component for cellular processes because its down regulation inhibits bacterial growth [3–5]. There are few reports where other chaperones such as trigger factors and DnaK were down regulated, but GroEL/ES were over expressed, leading to normal growth of the *E. coli* strain [6]. It was also discovered that GroEL plays an important role in cellular protein folding, prevention of aggregation of non-native proteins and assembly of lambda and T₄ bacteriophage [7,8]. GroEL is a tetradecameric protein having identical subunits. The molecular weight of each subunit is 57 kDa. These subunits assemble to form two heptameric rings which stack back to back forming a central cylindrical cavity [9]. This cylindrical structure of GroEL accommodates a substrate protein of approximately 60 kDa [10–13]. It is also able to fold large proteins by using the Trans mechanism of folding [14]. Each monomer subunit is organized in apical, intermediate and equatorial domains [9,10]. Apical domain is highly flexible, rich in hydrophobic amino-acid residues and forms the top of the central cylindrical channel in GroEL. The intermediate domain is rich in hydrophilic residues and makes the GroEL cavity charged to fold substrate proteins. Equatorial domains help in joining of monomeric subunits, providing an allosteric nature to the protein [9,10,15]. Different allosteric routes ensure the correct communication between subunits of the same and opposite ring [15]. The positive cooperative binding of ATP to one GroEL ring promotes GroES binding; converting it from a polypeptide accepting state to a folding active state while ATP binding to the opposite ring helps in the release of the GroES and folded protein from the GroEL cavity [11–13]. GroES is a seven membered ring composed of seven identical subunits of 10 kDa. It forms a stable complex with GroEL in the presence of Mg-ADP or Mg-ATP [13,15]. The apical domain of GroEL (191–376) and its C-terminal truncated fragment (191–345) shows very high chaperone like activity and is termed a minichaperone [16]. The N-terminal tail of this protein binds to the seven residues of substrate polypeptide by

Abbreviations: N, native; U, unfolded; I, intermediate; K_M, equilibrium constant from N → U; K_A, equilibrium constant from N → I; K_EQ, eq m I → U; Tm, melting temperature; ΔH^m, enthalpy change; ΔS^m, entropy change; ΔG^m, Gibbs free energy change; MaIZ, maltodextrin glucosidase; DTT, 1,4-dithiothreitol.

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hydrophobic and hydrogen bond interactions [16]. The minichaperone unfolds reversibly while the intact GroEL unfolds irreversibly on heating above 68 °C [16].

Monomeric GroEL is one subunit of the multimeric structure [17,18]. Like the tetradecameric GroEL and minichaperone, it helps in prevention of aggregation and folding of substrate protein. Monomeric GroEL folds non-native substrate in the absence of GroES and ATP [19]. It has a well-defined secondary, tertiary structure and is resistant to proteolysis [18,20,21]. The monomeric GroEL also unfolds reversibly [18,19]. It is known that a properly folded GroEL monomer undergoes assembly to form the tetradecameric structure [21–25]. The reassembly of monomeric subunits in the presence of ligands like MgCl₂, ATP, ADP, NaCl, ammonium sulphate, glycerol and co-chaperonin GroES is a two-step process which involves formation of the heptameric intermediate. This heptameric intermediate play a key role in the formation of tetradecameric GroEL protein [22]. Therefore, proper folding, conformation and stability of the GroEL monomer are very crucial factors to form functional GroEL protein [22,23]. So it is also important to know about the stability and folding of the GroEL monomer. Our approach is to study the unfolding pathway of monomeric GroEL by carrying out chemical denaturant and heat mediated equilibrium unfolding experiments. The folding/unfolding data can be used for calculation of thermodynamic stability parameters associated with the processes. The change in secondary and tertiary structure was measured by CD and intrinsic fluorescence based methods respectively. In case of GroEL monomer, we find that complete gain of secondary and tertiary structure occurs on dilution of denaturant. The urea induced unfolding of monomeric GroEL can be described as a three-state process which includes native, intermediate and unfolded states. The intermediate state populated at 2.0 M urea. As the process of equilibrium unfolding by urea is reversible, data is fitted to a three-state model for calculation of thermodynamic parameters (∆G∆H, ∆G∆H₂, ∆G∆H₂, ∆G∆H₂, ∆G∆H₂). The thermal unfolding studies from 20 to 90 °C with CD and fluorescence spectroscopy shows an unfolding transition from 40 to 60 °C and some residual structure unfolded at higher temperature. The denatured protein regains its structure on cooling from 90 to 20 °C. The thermal unfolding data was fitted to a two-state model. We were able to calculate its Tm from the transition curve while the Van’t Hoff’s plot was used for calculation of ∆H°m and ∆S°m.

2. Material and methods

2.1. Material

GroEL was cloned in the pACYC184 vector containing the GroEL gene. DH5α, BL21 E. coli strains were used for cloning, expression of GroEL. Urea was purchased from USB cooperation (USA). All experiments were performed in buffer having 20 mM tris, 100 mM (NH₄)₂SO₄, 10 mM MgCl₂ and 2 mM DTT at pH 7.5 Buffer was always prepared at room temperature.

2.2. Methods

2.2.1. Purification of GroEL, monomeric GroEL and MalZ proteins

GroEL was purified as previously with necessary modifications [9,30,31]. Transformed BL2–21 cells were grown in chloramphenicol containing Luria Broth at 37 °C and induced with 1 mM IPTG when the O.D reached 0.6. The cells were harvested by centrifugation at 8000 rpm for 20 min. The harvested cells were incubated in lysis buffer containing 50 mM tris, 1 mM DTT, 1 mg/ml lysozyme and 1 mM PMSF for 30 min. The cells were lysed by sonication and lystate was centrifuged at 10,000 rpm for one hour. Supernatant was collected and filtered through 0.45 um filter before applying to the chromatography column in an AKTA FPLC system. GroEL was purified with fast flow Q anion exchange and 15ISO hydrophobic interaction chromatography. The fraction collected from the hydrophobic interaction chromatography was treated with Affigel blue (Bio-Red) to remove bound proteins. The purified protein was stored in buffer containing 50 mM tris, 50 mM KCl, 1 mM DTT and 10% glycerol at 4 °C. The verification of purity was done on SDS PAGE gel (Fig. 1(A)) and tryptophan fluorescence on excitation at 295 nm and emission from 300 to 450 nm. The protein concentration was determined by taking absorbance at 280 nm using Beckman UV–vis spectrophotometer. Extinction coefficient (Aₐₜₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐ¢
Fig. 1. (A) SDS-PAGE shows purification of MalZ and GroEL protein. Lane 1 shows the medium range molecular weight marker; Lane 2, 3 purified MalZ (67 kDa) and lane 4, 5 purified GroEL (57 kDa). (B) Gel filtration elution profile of the monomeric GroEL by absorbance measurement at 280 nm. (C) Native Gel for the native and monomeric GroEL. Lane 1–5 shows the monomeric GroEL while lane 6 shows the native GroEL.

The protein concentration of native and monomeric GroEL was 0.1 uM and 2.5 uM respectively. Tertiary structures were compared by intrinsic tyrosine fluorescence spectroscopy. The protein was excited at excitation wavelength of 274 nm with slit width of 10 nm and fluorescence emission spectra were collected from 295 to 400 nm in 1 cm quartz cuvette. The protein concentration of native and monomeric GroEL was 0.1 uM and 2.5 uM respectively in buffer 20 mM tris, 100 mM (NH4)2 SO4, 10 mM MgCl2 and 2 mM DTT at pH 7.5.

2.2.5. Secondary structure determination of monomeric GroEL in different urea concentrations

The monomeric GroEL was incubated with different urea concentration (0–5 M) for one hour at 25 °C. The far-UV CD spectra of incubated samples were taken from a wavelength range of 240–210 nm. Each spectrum was corrected for contribution from buffer solution containing different urea concentrations. Secondary structure changes with respect to denaturant concentration were calculated by using Yang’s references tool. This tool is inbuilt with
the spectra-manager software available with J-815 CD spectrometer. It is based on the rotatory contribution of protein structure which can be represented by the equation: \[ \sum = x_i \xi_i \]. Where subscript \( i = H, \beta \) and R represent the fraction of helix, \( \beta \)-sheet and unordered form [29].

2.2.6. Equilibrium unfolding studies of monomeric GroEL by CD spectroscopy

For equilibrium unfolding studies, CD spectroscopy was performed using JASCO J-815 CD spectrometer flashed with nitrogen gas using an optical cuvette of path length 1 mm for measurement in far-UV region at 222 nm. The samples were prepared by incubating monomeric GroEL(2.5 uM) with different concentration of urea (0–6 M) from a stock of 10 M at 25°C for one hour in buffer 20 mM tris, 100 mM (NH₄)₂SO₄, 10 mM MgCl₂ and 2 mM DTT at pH 7.5. Each spectrum was corrected for contribution from buffer solution containing increasing concentration of urea (0–6 M). Mean residue ellipticity (MRE) at 222 nm was calculated using formula: \[ \theta = \frac{\theta_{obs}}{c} \text{Molar conc. of protein} \times \text{path length in mm} \times \text{number of amino acid residues in the protein} \] [32]. The obtained MRE data were plotted against various urea concentrations.

2.2.7. Spontaneous refolding studies of monomeric GroEL by CD spectroscopy

Highly concentrated protein was unfolded with 4 M urea at 25°C. Unfolded protein was diluted in a way resulting in final protein concentration of 2.5 uM and denaturant concentration being diluted from 4 M to 0.05 M in buffer 20 mM tris, 100 mM (NH₄)₂SO₄, 10 mM MgCl₂ and 2 mM DTT at pH 7.5. The equilibrated samples were excited at 274 nm and the emission spectra were recorded between 295 and 400 nm with excitation and emission slit width of 10 nm each. Each spectrum was obtained was subtracted from the buffer spectrum at that concentration of denaturant. To see the equilibrium transition, the relative fluorescence intensity at 307 nm and emission maximal wavelength \( \lambda_{max} \) was plotted against various denaturant concentrations.

2.2.8. Equilibrium unfolding studies of monomeric GroEL by intrinsic tryptophan fluorescence spectroscopy

As GroEL lacks tryptophan residues, tryptophan based intrinsic fluorescence was used for the urea denaturation based study of the monomeric GroEL. Samples were prepared on incubating protein (2.5 uM) with different concentration of urea (0–6 M) from a stock of 10 M at 25°C for one hour in buffer 20 mM tris, 100 mM (NH₄)₂SO₄, 10 mM MgCl₂ and 2 mM DTT at pH 7.5. The equilibrated samples were excited at 274 nm and the emission spectra were recorded between 295 and 400 nm with excitation and emission slit width of 10 nm each. Each spectrum was obtained was subtracted from the buffer spectrum at that concentration of denaturant. To see the equilibrium transition, the relative fluorescence intensity at 307 nm and emission maximal wavelength \( \lambda_{max} \) was plotted against various denaturant concentrations.

2.2.9. Spontaneous refolding studies of monomeric GroEL by CD and intrinsic tryptophan fluorescence spectroscopy

Highly concentrated protein was unfolded with 4 M urea at 25°C. Unfolded protein was diluted in a way resulting in final protein concentration of 2.5 uM and denaturant concentration being diluted from 4 M to 0.05 M in buffer 20 mM tris, 100 mM (NH₄)₂SO₄, 10 mM MgCl₂ and 2 mM DTT at pH 7.5. The refolding mixture was incubated for one hour at 25°C. The refolded protein was excited at 274 nm and emission fluorescence spectra were recorded at 295–400 ± 10 nm. Each spectrum was corrected for contribution from buffer solution containing increasing concentration of urea (0–6 M). To see the reversibility of the unfolding transition, the relative fluorescence intensity at 307 nm and the emission maximal wavelength \( \lambda_{max} \) of each refolded sample was plotted with the unfolding transition curve.

2.2.10. Data analysis

The urea induced equilibrium unfolding data obtained from CD and fluorescence spectroscopy was analyzed by drawing the baseline for the native, intermediate and unfolded states in the unfolding transition curve which can be further fitted to a three-state model [28]. The proposed equilibrium is

\[ N = I = U \]

\[ K_{NI} = \text{Equilibrium constant for } N = I \]

\[ K_{IU} = \text{Equilibrium constant for } I = U \]

\[ K_{NU} = \text{Equilibrium constant for } N = U \]

The observed signal of protein from both CD and fluorescence intensity of the protein \( S_{obs}(c) \) at any concentration of denaturant is given by the sum of the contribution of all the three states as:

\[ S_{obs}(c) = S_{N}f_{N}(c) + S_{I}f_{I}(c) + S_{U}f_{U}(c) \]  

(1)

\[ f_{N}(c), f_{I}(c), f_{U}(c) \] are the fractions of three states at a urea concentration \( c \) and \( S_{N}, S_{I} \) and \( S_{U} \) are the signal for pure N, I and U states respectively. The fractions \( f_{N}, f_{I} \) and \( f_{U} \) are related to \( K_{NI} \) and \( K_{IU} \) of unfolding transition from \( N \equiv I \) and \( N \equiv U \) respectively, hence are related to corresponding free energy changes \( \Delta G_{NI} \) and \( \Delta G_{NU} \), as follows:

\[ f_{N} = \frac{1}{1 + K_{NI} + K_{NU}} = \frac{1}{1 + \exp(-\Delta G_{NI}/RT)} + \exp(-\Delta G_{NU}/RT) \]

\[ f_{I} = \frac{K_{NI}}{1 + K_{NI} + K_{NU}} = \exp(-\Delta G_{NI}/RT) \cdot [1 + \exp(-\Delta G_{NU}/RT)] \]

\[ f_{U} = \frac{K_{NU}}{1 + K_{NI} + K_{NU}} = \exp(-\Delta G_{NI}/RT) \cdot [1 + \exp(-\Delta G_{NU}/RT)] \]

(2)

(3)

\[ \Delta G_{NI} = \Delta G_{N}^{H_{2}O} - m_{NI}c \]

\[ \Delta G_{N}^{H_{2}O} = \Delta G_{N}^{H_{2}O} - m_{NI}c \]

\[ \Delta G_{NU} = \Delta G_{N}^{H_{2}O} - m_{NU}c \]

\[ \Delta G_{NI}^{H_{2}O} \text{ and } \Delta G_{NU}^{H_{2}O} \text{ are } \Delta G_{NI} \text{ and } \Delta G_{NU} \text{ at } 0 \text{ M urea. } m_{NI} \text{ and } m_{NU} \text{ represents the dependence of respective free energy changes on denaturant concentration(c) and the co-operativity of transition. So from the Eqs. (1)–(3), } S_{obs}(c) \text{ is given by:} \]

\[ S_{obs}(c) = S_{N} + S_{I}\exp(-\Delta G_{NI}^{H_{2}O}/RT) + S_{U}\exp(-\Delta G_{NU}^{H_{2}O}/RT) \cdot [1 + \exp(-\Delta G_{NI}^{H_{2}O}/RT) + \exp(-\Delta G_{NU}^{H_{2}O}/RT)] \]

(4)

In general \( S_{N}, S_{I} \) and \( S_{U} \) are dependent on the concentration of denaturant and we assume a linear dependence on the concentration of denaturant (c) as \( S_{N} = a_{1} + b_{1}c, S_{I} = c_{1} + p_{1}c \) and \( S_{U} = e_{1} + g_{1}c \) where \( a_{1}, b_{1}, c_{1}, p_{1}, e_{1} \) and \( g_{1} \) are constants. \( a_{1}, b_{1}, c_{1}, p_{1}, e_{1}, g_{1} \) were calculated from intercept and slope of baseline of the native, intermediate and unfolded states. By using all the above equations,
the urea mediated equilibrium unfolding data was analyzed and
thermodynamic parameters of stability were calculated.

2.2.11. Thermal unfolding of monomeric GroEL by CD and
fluorescence spectroscopy

For CD spectroscopy based thermal denaturation studies, the
monomeric GroEL solution (2.5μM) was prepared in buffer 20 mM
tris, 100 mM (NH₄)₂ SO₄, 10 mM MgCl₂ and 2 mM DTT at pH 7.5
and placed in 1 mm quartz cuvette. The sample was heated from
20 to 90 °C with a heating rate of 1 °C/min controlled by a Jasco-815
programmable peltier element. A continuous scan at a wavelength
of 222 nm was collected for each temperature. Mean Residual Ellip-
ticity (MRE) was calculated from the millidegree values and plotted
against the various temperatures. The reversibility of the thermal
unfolding process is more in tris buffer as compared to phosphate
buffer hence tris buffer was selected for the thermal studies. To
minimize the heating effect on tris, the buffer was always prepared
freshly at room temperature.

For the fluorescence based thermal denaturation studies, the
monomeric GroEL solution (2.5μM) in 1 cm quartz cuvette was
placed in a thermostatic holder of the spectrofluorimeter. The
holder was maintained at a constant temperature by circulating
water from a constant temperature water bath. After a set temper-
ature was reached, the protein was excited at wavelength 274 nm
and a continuous emission scan was collected at 307 nm from tem-
perature 20–90 °C. The fluorescence intensity was plotted against
the temperature from 20 to 90 °C.

The stepwise heat denaturation experiment was also performed
by intrinsic tyrosine fluorescence spectroscopy. The monomeric
GroEL solution of concentration 2.5 μM was incubated at different
temperature from 20 to 90 °C for 5 min before taking an emission
spectrum. Each incubated sample was excited at 274 ± 10 nm and
emission spectra were collected from 295 to 400 ± 10 nm at all
temperatures.

2.2.12. Thermal refolding of monomeric GroEL by CD and
fluorescence spectroscopy

For far-UV CD spectroscopy based thermal refolding studies, 90 °C
heat denatured monomeric GroEL solution (2.5μM) was gradu-
ally cooled from 90 to 20 °C with a cooling rate of 1 °C/min
controlled by a Jasco-815 programmable peltier element. A con-
tinuous scan at a wavelength of 222 nm was collected for each
temperature from 90 to 20 °C. The MRE value was calculated from
millidegree data as mentioned above and plotted against the vari-
ous temperatures.

For the fluorescence based thermal refolding studies, 90 °C
heat denatured monomeric GroEL solution (2.5μM) was gradu-
ally cooled from 90 to 20 °C. The denatured protein was excited at wave-
length 274 nm and a continuous emission scan was collected at
307 nm from temperature 90–20 °C. The fluorescence intensity was
plotted against the various temperatures from 90 to 20 °C.

The step wise refolding experiment was also performed by
intrinsic tyrosine fluorescence spectroscopy. The monomeric GroEL
solution of concentration 2.5 μM was incubated at 90 °C for 5 min
and then gradually cooled. As it cooled, the intrinsic tyrosine emis-
ション spectra were collected at different temperatures from 90
to 20 °C. Each incubated sample was excited at 274 ± 10 nm and
emission spectra were collected from 295 to 400 ± 10 nm at all
temperatures.

2.2.13. Data analysis

Reversible thermal denaturation was analyzed by fitting the
thermal denaturation transition to a two-state model. A two-state
denaturation equilibrium means only native (N) and denatured (D)
states of a protein are significantly populated in the transition pro-
cess. So equilibrium for this process can be represented by Keq:

$$Keq = [D]/[N]$$  \hspace{1cm} (5)

We can create Van’t-Hoff’s plot to relate Keq to the temperature

$$\ln Keq = -\Delta H/R (1/T) + \Delta S/R$$  \hspace{1cm} (6)

$$S_{obs} = S_N + S_0 \exp [-(\Delta G_{ND}/RT)]/1 + \exp [-(\Delta G_{ND}/RT)]$$  \hspace{1cm} (7)

Here, we assumed $S_N$ and $S_0$ are linearly dependent on the temper-
ature so $S_N = a_1 + b_1 T$, $S_0 = c_1 + p_1 T$, Where $a_1$, $b_1$, $c_1$ and $p_1$
are constants obtained from the intercept and slope of native and
unfolded baseline respectively. By using unfolding transition curve
and Van’t-Hoff’s plot we had calculated the $T_m$, $\Delta H_{avm}$ and $\Delta S_{avm}$
for the monomeric GroEL. Gibbs-Helmoltz equation was not used
for the fitting of thermal unfolding data. For the fitting of thermal
unfolding data in this equation $\Delta C_p$ is required. The value of $\Delta C_p$
can be experimentally calculated by using calorimetry. The lim-
itation of this technique is that, for calculation of errorless $\Delta C_p$
it requires high concentration of protein i.e above 1 mg/ml. But
monomeric GroEL have tendency to form concentration dependent
oligomers at protein concentration of 1 mg/ml. Hence we used Eq.
(7) for the fitting of thermal unfolding transition.

3. Results

3.1. GroEL monomer purification

Urea denatured native GroEL was injected into HPLC-Tosho
SWXL 4000 size-exclusion column and eluted at a flow rate of
0.5 ml/min with 20 mM tris, 100 mM (NH₄)₂ SO₄, 10 mM MgCl₂
and 2 mM DTT at pH 7.5 buffer. The monomeric GroEL peak at 280 nm
was obtained at an elution volume of 13.5 ml or 26 min (Fig. 1(B)).
The verification of GroEL monomer was done using native PAGE gel.
Lane 1–5 in (Fig. 1(C)) shows the band of the monomeric GroEL and
lane 6 shows the band of the native GroEL. Native PAGE Gel also
proves that the monomeric GroEL preparation is not contaminated
with the native GroEL.

3.2. Monomeric GroEL prevents aggregation of substrate protein
MalZ

The effect of monomeric GroEL on the aggregation prevention
of MalZ was measured by using light scattering kinetics. In Fig. 2, the
denatured MalZ protein diluted in buffer showed the highest light
scattering meaning the highest aggregation. All other light scatter-
ing data in presence of BSA and monomeric GroEL were plotted
relative to it. The light scattering profile in the presence of BSA
is similar to the buffer diluted MalZ. This shows that BSA is not
playing any significant role in the aggregation prevention of MalZ
protein. When denatured MalZ was diluted in different molar ratios
of monomeric GroEL, it shows a decrease in light scattering in a
concentration dependent manner. An increase in molar excess of
monomeric GroEL in the dilution mixture leads to enhanced
prevention of aggregation of MalZ protein. A 1:5 molar ratio of MalZ:
monomeric GroEL shows only 10–15% prevention of aggregation,
1:10 molar ratio of MalZ: monomeric GroEL shows 25–30% pre-
vention while molar ratio of 1:14 of MalZ: monomeric GroEL shows
approximately 60% prevention of aggregation. The pattern of aggre-
gation prevention of molar ratios 1:14 and 1:15 are similar, showing
that a further increase in monomeric GroEL concentration doesn’t
show any change in aggregation profile.
3.3. Time course of folding of denatured MalZ

MalZ is not a reversible refoldable protein, while it regains almost 50% of its activity by refolding in the presence of GroEL+GroES+ATP. In this experiment the molar ratios of MalZ:GroEL:GroES was taken as 1:2:5. The ATP concentration used was 5 mM [26]. Its spontaneous folding is only 5–10% [26]. To see the effect of monomeric GroEL on the folding of denatured MalZ, it was diluted 100 fold with dilution buffer and buffer containing different molar ratios of monomeric GroEL mentioned in methods. In Fig. 3 the
activity of native MalZ was taken as 100%. Regained activities of denatured MalZ in presence of different molar ratios of monomeric GroEL were plotted with respect to activity of native protein. The regain activity of spontaneous refolded MalZ is found to be only 10% while in different molar ratios 1:1.0 and 1:1.4 (MalZ: monomeric GroEL) shows 15 and 25% regain of activity after 60 min reactivation time.

3.4. Secondary and tertiary structure comparison between tetradecameric and monomeric GroEL

The secondary structures of native and monomeric GroEL were compared by taking CD spectra from a wavelength 200–240 nm. The CD data obtained was plotted as MRE vs wavelength for both the native and monomeric GroEL as shown in Fig. 4(A). These CD spectra were used for calculation of percentage of helix, sheet, turn and random structure for native and monomeric GroEL by the method of Chen [30]. The percentage of helix, sheet, turn and random structures are approximately 33.7%, 24.9%, 12.9% and 28.5% respectively in native GroEL while in monomeric GroEL the percentage of helix, sheet, turn and random structures are 26.9, 29.7%, 9.1% and 34.2% respectively as shown in Table 1.

To compare the tertiary structure of proteins, intrinsic tyrosine emission fluorescence was used. Fig. 4(B) shows the emission spectra of both native GroEL and monomeric GroEL from 295 to 400 nm. The pattern of emission from both the proteins is same while spectra vary in intensity of emission. Fig. 4(C) shows the emission spectra of monomeric GroEL. The absence of tryptophan is also evident in the emission fluorescence spectra of both native and monomeric GroEL protein.

3.5. Secondary structure comparison of monomeric GroEL in different denaturant concentrations

CD Spectra of monomeric GroEL (2.5 μM) in the presence of different urea concentrations (0–5 M) were recorded at 25 °C in the far-UV wavelength range from 240 to 210 nm. In Fig. 5 MRE values obtained from far-UV CD spectra of monomeric GroEL in different urea concentrations is plotted with wavelength range 240–210 nm. Few conformational changes occur at low denaturant concentration. MRE spectra have a more negative value at the urea concentration of 0.1–0.3 M as compared to native protein. This shows the gain of few secondary structures at lower urea concentration 0.1–0.3 M urea while decrease in content of secondary structure on further increase of urea concentration. Complete loss of secondary structure occurs at 4.0 M urea concentration. Percentage of secondary structure in term of helix, sheet, turn and random was calculated using Yang’s reference software mentioned in methods. The percentage secondary structure at different urea concentrations is shown in Table 2.

3.6. Equilibrium unfolding and refolding of monomeric GroEL monitored by CD spectroscopy

The change in ellipticity at 222 nm was measured at different urea concentrations from 0–6 M. The MRE values calculated from far-UV spectra at 222 nm corresponding to each sample were plotted against respective urea concentration from 0 to 6 M. This is known as an unfolding transition curve. The MRE value is more negative at lower denaturant concentration 0.1–0.3 M urea which shows a gain in secondary structures at lower urea concentration 0.1–0.3 M. Then there is a gradual decrease, leading to unfolding of protein on increasing the denaturant concentration. 4.0 M of urea is able to unfold the protein completely (Fig. 6).

During refolding studies, the unfolded protein was diluted with refolding buffer up to the minimum possible denaturant remains in solution. The final protein concentration was 2.5 μM as used for unfolding experiments. The buffer diluted protein is able to regain its secondary structure on incubation for one hour (Fig. 6). This shows that protein have tendency to refold in a reversible manner. The MRE data calculated from m−degree values obtained from CD spectra for unfolding and refolding were plotted against urea concentration. The MRE value corresponds to 0.1 M urea is omitted in the unfolding transition curve for the data fitting. The native, intermediate and unfolded state baselines are drawn. The small-dashed, long-dashed and solid black line shows baseline of the native, the intermediate and unfolded states respectively. So the unfolding process can be shown as N—⇔I—⇔U. The slope and intercept helps in calculation of m (cooperativity) and Cm (mid-point of unfolding) which was used for calculation of ΔG_{U}^{\text{H,O}}, ΔG_{IU}^{\text{H,O}} and ΔG_{NU}^{\text{H,O}} (Table 3).

3.7. Equilibrium unfolding and refolding of monomeric GroEL monitored by fluorescence spectroscopy

The urea induced equilibrium unfolding of the monomeric GroEL (2.5 μM) was studied by intrinsic tyrosine fluorescence. Fig. 7(A) shows that the emission spectra of native and refolded protein. The similarity in the fluorescence intensity of native and refolded protein proved that the protein has a tendency to refold reversibly. The λ_{max} is not similar for the native and refolded protein which shows that even though protein regains its tertiary structure but the conformation of the refolded protein is not similar to the native protein.

Fig. 7(B) shows the fluorescence intensity change at 307 nm and change in λ_{max} in presence of increasing concentration of urea. The relative fluorescence intensity transition curve shows that at 0.3 M concentration of urea, the tyrosine fluorescence emission intensity is higher than native but decreases gradually from 0.5 to 4 M of urea. There is no major change in fluorescence intensity from 4 to 6 M concentration of urea. The unfolding induced by urea exhibited two transition processes. During the first transition, fluorescence intensity decreases after 0.3 M urea concentration while a slight red shift of λ_{max}. The small red shift shows that tyrosine environment is not appreciably altered. During the second transition, further denaturation process was accompanied by a decrease in fluorescence intensity and large red shift of λ_{max}. After 4.0 M urea concentration protein undergoes complete unfolding as no further apparent changes were observed in both fluorescence intensity and λ_{max}.

During refolding studies, unfolded monomer was diluted in refolding buffer up to a maximum possible dilution of 0.05 M and incubated for one hour. Fig. 7(B) shows the relative fluorescence intensity and the λ_{max} upon refolding of the protein. The fluorescence intensity of the refolded protein is similar to the native protein which shows that protein regains its tertiary structure upon removal of denaturant. The pattern of λ_{max} of refolding transition is similar to the λ_{max} of unfolding transition but the value λ_{max} for the refolded protein is higher than the native protein. It shows that the refolded protein have more exposed tyrosine residue than the native protein. The reason for the change for conformation of protein can be presence of residual urea in refolding mixture.

In Fig. 7(C) the long-dashed, small-dashed and solid black line shows the baseline of native, the intermediate and unfolded states respectively. An unfolding and refolding process can be shown as N—⇔I—⇔U. The slope and intercept helps in calculation of m (cooperativity) and Cm (mid-point of unfolding) which was used for calculation of ΔG_{U}^{\text{H,O}}, ΔG_{IU}^{\text{H,O}} and ΔG_{NU}^{\text{H,O}} (Table 3).
Fig. 4. CD and fluorescence spectra of the native and monomeric GroEL. Panel (A) shows the CD spectra of monomeric (2.5uM) and native GroEL (0.1uM). (○) shows MRE spectrum of native GroEL and (*) shows MRE spectrum of monomeric GroEL. Panel (B) shows the intrinsic tyrosine emission fluorescence spectra of the monomeric GroEL and the native GroEL. Spectra in dark circle (•) represent fluorescence emission spectrum of monomeric GroEL. Spectra in open circle (○) represent fluorescence emission spectrum of native GroEL. Panel (C) shows the intrinsic tyrosine emission fluorescence spectra of the monomeric GroEL separately.

Table 1
Percentage secondary structure of native and monomeric GroEL from the spectra obtained from 240 to 200 nm scan by using Yang’s references software.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Protein</th>
<th>Helix (%)</th>
<th>Sheets (%)</th>
<th>Turn (%)</th>
<th>Random (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GroEL</td>
<td>33.7</td>
<td>24.9</td>
<td>12.9</td>
<td>28.5</td>
</tr>
<tr>
<td>2</td>
<td>GroEL monomer</td>
<td>26.9</td>
<td>29.7</td>
<td>9.1</td>
<td>34.2</td>
</tr>
</tbody>
</table>

Table 2
Percentage secondary structures of monomeric GroEL at various concentrations of urea (0–5 M) were calculated from the spectra shown in Fig. 5a by using Yang’s reference software.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Urea Conc. [M]</th>
<th>Helix (%)</th>
<th>Sheets (%)</th>
<th>Turn (%)</th>
<th>Random (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>18.2</td>
<td>23.2</td>
<td>23.6</td>
<td>35.0</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>21.9</td>
<td>31.0</td>
<td>14.4</td>
<td>32.7</td>
</tr>
<tr>
<td>3</td>
<td>0.3</td>
<td>18.5</td>
<td>20.6</td>
<td>20.9</td>
<td>40.0</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>9.2</td>
<td>13.3</td>
<td>26.9</td>
<td>50.6</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>0.5</td>
<td>6.7</td>
<td>37.3</td>
<td>55.5</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>0.0</td>
<td>4.8</td>
<td>40.0</td>
<td>55.2</td>
</tr>
</tbody>
</table>
Fig. 5. Far-UV CD spectra of the monomeric GroEL in presence of different urea concentrations: Far UV-CD spectra of (•) native state; (○) 0.3 M urea; (▼) 1.0 M urea; (△) 4.0 M urea and (■) 5.0 M urea. Monomeric GroEL undergoes complete unfolding at 4.0 M urea concentration.

Fig. 6. Equilibrium unfolding and refolding of monomeric GroEL measured by CD spectroscopy: Equilibrium unfolding of monomeric GroEL was monitored by far UV-CD spectroscopy. Unfolding data points are shown as (●) black line while refolding data points are represented as (Grey square). The native, intermediate and unfolded state baselines are drawn. Small dotted, long dashed and dark black line shows the baseline of native, intermediate and unfolded states respectively. Intercept and slope of all the baselines were used for the data fitting in Eq. (4).

Table 3
Thermodynamic fits of urea mediated unfolding transition data.

<table>
<thead>
<tr>
<th>Probe</th>
<th>$m_{0}$ kcal/mol</th>
<th>$\Delta G_{m}^{\text{H}_{2}O}$ kcal/mol</th>
<th>$m_{0}$ kcal/mol M</th>
<th>$\Delta G_{m}^{\text{H}_{2}O}$ kcal/mol</th>
<th>$m_{0}$ kcal/mol M</th>
<th>$\Delta G_{m}^{\text{H}_{2}O}$ kcal/mol</th>
<th>$C_{m}$ M</th>
<th>$\Delta G_{m}^{\text{H}_{2}O}$ kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>1.00 ± 0.6</td>
<td>7.10 ± 1.0</td>
<td>2.22 ± 0.1</td>
<td>2.35 ± 1.0</td>
<td>2.3 ± 0.5</td>
<td>1.03 ± 0.1</td>
<td>4.74 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>Intrinsic Fluorescence</td>
<td>0.98 ± 0.5</td>
<td>7.12 ± 1.0</td>
<td>3.18 ± 0.3</td>
<td>2.24 ± 0.1</td>
<td>2.36 ± 1.0</td>
<td>1.05 ± 0.3</td>
<td>4.80 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>
\[ \Delta G_{\text{NU}}^{H_2O} \] at zero denaturant concentration is \( 7.12 \pm 1.0 \text{ kcal/mol} \). The corresponding m\(_{\text{NU}}\) value is \( 3.18 \pm 0.3 \text{ kcal/mol.M} \) and Cm\(_{\text{NU}}\) is \( 2.24 \pm 0.1 \text{ M} \).

From the theoretical curves, the apparent fraction of native \( (f_N) \), intermediate \( (f_I) \) and unfolded \( (f_U) \) protein with respect to denaturant concentration calculated by using Eq. (2) (Fig. 8(A)). The maximum population of intermediate state are observed at 2.0 M urea concentration which is about 85%. Theoretical curves in Fig. 8(B) shows the free energy change extrapolated to zero urea concentration. The values of \( \Delta G_{\text{NI}}^{H_2O} \), \( \Delta G_{\text{NU}}^{H_2O} \) and \( \Delta G_{\text{NU}}^{H_2O} \) are \( 2.35 \pm 1.0 \text{ kcal/mol}, 4.75 \pm 1.0 \text{ kcal/mol} \) and \( 7.10 \pm 1.0 \text{ kcal/mol} \) respectively. This shows that a stable intermediate is formed in the unfolding process.

3.8. Thermal unfolding and refolding studies of monomeric GroEL by using CD spectroscopy

Thermal unfolding and refolding of the monomeric GroEL was monitored by far-UV CD and tyrosine emission fluorescence spectroscopy. The unfolding transition of monomeric GroEL with temperature \((20\text{–}90 \degree \text{C})\) monitored at 222 nm is a sigmoidal curve. Fig. 9 shows that secondary structure remains the same up to 38 \degree \text{C}, gradually decreasing up to 60 \degree \text{C} and complete loss of secondary structure after 75 \degree \text{C}. This shows that the protein undergoes complete unfolding at 75 \degree \text{C}.

The 90 \degree \text{C} heat denatured protein was cooled from 90 to 20 \degree \text{C} and it regained its secondary structure. This shows process is reversible. The native and unfolded baselines are drawn in Fig. 9.
Fig. 8. Theoretical curve showing different population during unfolding pathway and free energy extrapolated to zero urea concentration: Panel ‘A’ shows the urea concentration dependence of populations of native (●, Δ), intermediate (○, ■) and unfolded state (×, □) of monomeric GroEL calculated from the thermodynamic parameters listed in Table 3. The maximum fractional population of I state are observed at 2 M urea which is about 85%. Panel ‘B’ shows the free energy values extrapolated to zero urea concentration. ● and Δ represent the extrapolation line for ΔG\text{GU} \text{H}_2\text{O} and ○ shows the extrapolation line for ΔG\text{GU} \text{H}_2\text{O} and ■ shows the extrapolation line for ΔG\text{GU} \text{H}_2\text{O} obtained from the CD and fluorescence data respectively. The thermodynamic parameters obtained from best fitted data are shown in Table 3.

Table 4
Thermodynamic fits of thermal unfolding transition.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Probes</th>
<th>ΔH\text{mon}(kcal/mol)</th>
<th>ΔS\text{mon}(cal/mol/K)</th>
<th>ΔG\text{mon(H2O)} kcal/mol</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CD data</td>
<td>43.3 ± 0.1</td>
<td>143.9 ± 0.1</td>
<td>7.0 ± 1.0</td>
<td>46.0 ± 1.0</td>
</tr>
<tr>
<td>2</td>
<td>Intrinsic Fluorescence</td>
<td>42.7 ± 0.1</td>
<td>142.0 ± 0.1</td>
<td>6.8 ± 1.5</td>
<td>46.8 ± 1.0</td>
</tr>
</tbody>
</table>

Fig. 9. Thermal unfolding and refolding transition curve measured by CD spectroscopy: Thermal unfolding and refolding transition curve of the monomeric GroEL measured by CD spectroscopy at 222 nm from a temperature range of 20–90 °C. Black line represents the unfolding transition while grey line represents the refolding on cooling from 90 to 20 °C. Dashed and dash-and-dot line shows the baseline of native and unfolded state respectively. Intercept and slope of baselines were used for the fitting of data in Eq. (7).

Dashed and dash-and-dotted line shows the baseline for native and unfolded states respectively. The data was fitted to a two-state model (Eq. (6)). The parameters obtained from the transition curve and fitting helps in calculation of Tm and ΔG\text{GU} \text{H}_2\text{O}. Table 4 shows the Tm of monomeric GroEL is 46.0 ± 1.0 °C and ΔG\text{GU} (25 °C) is 7.0 ± 1.0 kcal/mol.

The thermal unfolding and refolding of protein was also studied by stepwise heating and cooling from 20 to 90 °C with excitation and emission wavelength of 274 ± 10 nm and emission spectra were collected from 295 to 400 nm. This also shows the reversibility of unfolding and refolding process (Fig. 10A, B).

3.9. Thermal unfolding and refolding studies of monomeric GroEL by using fluorescence spectroscopy

Change in tertiary structure of the monomeric GroEL with temperature was also monitored by recording changes in intrinsic tyrosine fluorescence intensity with continuous scan from 20 to 90 °C. There is a continuous decrease in tyrosine fluorescence from 20 to 90 °C. In the same way 90 °C unfolded protein was cooled down from 90 to 20 °C to observe the regain of tertiary structure of protein. Here also, unfolding process is found reversible (Fig. 10C). The native and unfolded states baselines are drawn in Fig. 10C. The long dashed and small dashed line shows the baseline of native and unfolded states respectively. The data was fitted to a two-state model (Eq. (6)). The parameters obtained from the transition curve and fitting helps in calculation of Tm and ΔG\text{GU} \text{H}_2\text{O}. Table 4 shows the Tm of monomeric GroEL is 46.8 ± 1.0 °C and ΔG\text{GU} (25 °C) is 6.8 ± 0.5 kcal/mol.

In Figs. 9 and 10C it is also noticeable that the MRE value is little more negative and emission fluorescence value is little bit higher at 70–75 °C. This shows that some residual structures are unfolded at high temperature but major transition is from 40 to 60 °C. Due to one prominent transition, data was best fit to a two-state model.

Fig. 10(D) shows the Van’t Hoff’s plot to relate K\text{eq} to the temperature. The lnK\text{eq} obtained from Eq. (5) was plotted against the reciprocal of temperature. The slope and intercept of the plot are used for calculation of ΔH\text{mon} and ΔS\text{mon} respectively. The values of ΔH\text{mon} and ΔS\text{mon} correspond to CD data are 43.3 ± 0.1 kcal/mol and 143.9 ± 0.1 cal/mol/K respectively. The val-
values of $\Delta H^{\text{van}}$ and $\Delta S^{\text{van}}$ corresponds to intrinsic fluorescence data are 42.7 ± 0.1 kcal/mol and 142.0 ± 0.1 cal/mol/k respectively.

4. Discussion

Equilibrium unfolding and refolding of the monomeric GroEL have been studied by CD and fluorescence spectroscopy. From the urea mediated equilibrium unfolding studies, two transitions were obtained, one at lower urea concentration while another at urea concentration of 2.0 M urea. The protein becomes fully unfolded at 4.0 M urea concentration (Figs. 6 and 7 B). Due to the presence of more than one kind of observed population in the process, the data was fitted to a three-state model which explains the presence of N, I and U populations in the unfolding process (Figs. 6 and 7 B). Native protein population exists below 2.0 M urea concentration, intermediate population is largely at the urea concentration of 2.0 M urea (Fig. 8A). From this data, it is reasonable to assume the existence of a three state mechanism in which a stable intermediate is populated during the equilibrium unfolding process at 2.0 M concentration of urea (Fig. 8A). The percentage of intermediate state at 2.0 M urea concentration is about 85% (Fig. 8A).

During the heat mediated unfolding process, a prominent transition during equilibrium unfolding of monomeric GroEL had occurred from 40 to 60 °C. The range of the second transition was very narrow so it’s not possible to fit it to a three-state model, but it clearly showed that some residual structure unfold near 70 °C. Protein undergoes complete unfolding after 75 °C (Figs. 9 and 10 C). To correlate the loss of fluorescence signal of monomeric GroEL with the thermal unfolding process, it is worth mentioning that the decrease in fluorescence intensity and loss of secondary structure (monitored by CD spectroscopy) are happening upon heating the protein solution. During the fitting of data from thermal denaturation process, the data from both CD and fluorescence signals were normalised and globally fitted in the two-state model. The thermal denaturation is also reversible.

In the previous studies, equilibrium unfolding and stability of GroEL minichaperones, apical domain with C-terminal domain (191–376) and isolated apical domain (191–345) were compared. Minichaperone (191–345) is only the apical domain while (191–376) includes apical domain core and C-terminal domain. Thermal denaturation experiments suggested similar midpoints of denaturation for GroEL (191–345) and GroEL (191–376) when
probed with fluorescence spectroscopy which indicated that the tertiary structure, of the two, melted exactly at the temperature corresponding to unfolding of the apical domain core. Unfolding of domain core is reversible in presence or absence of the C-terminal domain. In the minichaperone (191–376) there is a well separated low temperature transition during equilibrium unfolding which corresponds to the C-terminal domain. The bis-ANS based urea mediated unfolding studies showed that, at post-transition region; an intermediate is populated with an exposed hydrophobic surface. This was confirmed by an increase in fluorescence intensity on binding of bis-ANS to exposed hydrophobic residues. This intermediate has been attributed to the apical domain [16].

A comparison of the above study with the present monomeric GroEL unfolding data suggests that; first transition in urea mediated unfolding of monomeric GroEL may correspond to unfolding of its intermediate and equatorial domain while the stable apical domain undergoes unfolding process at higher urea concentration (2 M). This represents the apparently independent unfolding transition of two or more domains of the large multi-domain proteins. The complete unfolding of protein occurs at 4.0 M urea. From this process, it is clear that one intermediate has formed having a folded apical domain and an unfolded intermediate and equatorial domain. Thus, the proposed mechanism is:

\[
\begin{align*}
K_{\text{N}} & \quad N \\
K_{\text{U}} & \quad I \\
K_{\text{IU}} & \quad U
\end{align*}
\]

Where \( K_{\text{N}}, K_{\text{U}}, \) and \( K_{\text{IU}} \) are the equilibrium constants for the \( N = I, I = U, \) and \( N = U \) transitions, respectively.

During the thermal unfolding of monomeric GroEL, a prominent transition occurs from 40 to 60 °C as shown in Figs. 9 and 10 (C). Folding/unfolding processes are reversible and data are fitted to a two-state model. By comparing our present results with earlier minichaperone studies, we can conclude that the first major transition from 40 to 60 °C may correspond to unfolding of intermediate and equatorial domain while a small transition at higher temperature may be representative of unfolding of the stable residual structure of the apical domain (Figs. 9 and 10 c).

Along with this, thermal and urea induced unfolding studies allowed us to determine the thermodynamic stability parameters of GroEL monomer in terms of \( T_m, \Delta H^{\text{unf}} \) and \( \Delta S^{\text{unf}} \) and \( \Delta G_{\text{N}}^{\text{H}_2O} \) at 25 °C. \( \Delta G_{\text{N}}^{\text{H}_2O} \) calculated from both the transition curves coincide with each other. The value of \( \Delta G_{\text{N}}^{\text{H}_2O} \) from urea and thermal unfolding is 7.10 ± 1.0 kcal/mol and 7.01 ± 1.0 kcal/mol, respectively (Table 3, 4). The \( T_m, \Delta H^{\text{unf}} \) and \( \Delta S^{\text{unf}} \) of the protein is 46.0 ± 0.5 °C, 43.3 ± 0.1 kcal/mol and 143.0 ±0.1 cal/mol/k respectively (Table 4).

In the \( E. \) coli proteome it had been found that approximately 15% of proteins have a free energy change of \( \leq 40 \) kcal/mol while it undergoes an abrupt decrease once the cell is in thermal stress [27]. Hence, the above calculated values of thermodynamic stability parameters for monomeric GroEL reflect that it is a reasonably stable entity (Table 3, 4) and it can be expected that a tetradesamicer assembly constituted of stable monomeric subunits would provide an even greater stability against thermal and chemical perturbations.

It is reported in the literature that monomeric GroEL possesses chaperone like activity to fold small substrate proteins in the absence of GroES and ATP [19]. A majority of proteins in the cell belong to the class of large multidomain family which often unfold irreversibly in vitro due to the presence of competing side reactions such as aggregation and misfolding [33]. So chaperone assisted folding is a good option to fold the large and multi-domain proteins. Here we explored the aggregation prevention and folding of a large substrate protein MalZ, an \( E. \) coli cytosolic protein of 67 kDa, in presence of monomeric GroEL. A good preparation and stability under \textit{in vitro} conditions must be optimised for proper functioning of monomeric GroEL for the folding of the substrate protein. Our results reflect that it is helping in prevention of aggregation and folding of urea denatured substrate. The folding of denatured protein was confirmed by its activity assay and we found that denatured protein regained 30% of its activity in the presence of monomeric GroEL, while the spontaneous refolding of denatured protein is only 10%. The percentage regain of activity was calculated with respect to activity of native protein (Figs. 2, 3). From here we can conclude that GroEL monomer also exhibits chaperone like activity towards large multi-domain aggregation prone substrate proteins.

Thus, the outcome of the present study involving the folding/unfolding pathways, determination of thermodynamic stability, investigation of the chaperone/chaperone like activity of monomeric GroEL, provides us preliminary information about the stability and mechanistic aspects of folding by the building block of native GroEL. Taking into consideration the present studies, and further careful investigation in the near future it would certainly provide much focused information on GroEL stability.

Author contributions

S.P, T.K.C designed the experiments. S.P conducted the experiments. S.P, T.K.C analyzed the data. S.P, T.K.C wrote and edited the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jibiomac.2016.12.054.

Supporting information includes the size-exclusion elution profile of tetradesamicer GroEL, global fitting of the urea and heat mediated unfolding transition curves of monomeric GroEL as Figs. S1, S2 and S3 respectively. The parameters obtained from the best fit were further used for calculation of thermodynamic parameters of stability for the monomeric GroEL protein.

References


