Protein Engineering vol.14 no.6 pp.391–395, 2001

COMMUNICATION

Metal ions modulate the plastic nature of *Mycobacterium tuberculosis* chaperonin-10

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Chaperonin-10s possess a highly flexible segment of ~10 residues that covers their dome-like structure and closes the central cavity of the chaperonin assembly. The dome loop is believed to contribute to the plasticity of their oligomeric structure. We have exploited the presence of a single tryptophan residue occurring in the dome loop of *Mycobacterium tuberculosis* chaperonin-10 (cpn-10), and through intrinsic fluorescence measurements show that in the absence of metal ions, the tryptophan is almost fully solvent exposed at neutral pH. The dome loop, however, assumes a closed conformation in the presence of metal ions, or at low pH. These changes are fully reversed in the presence of chelating agents such as EDTA, confirming the role of cations in modulating the metastable states of cpn-10.
Keywords: chaperonin-10/dome loop/metastable state/*Mycobacterium tuberculosis*/structural plasticity

Introduction

Chaperonins are among the best-characterized classes of molecular chaperones. *Escherichia coli* possesses a larger 60 kDa chaperonin, the GroEL, and a smaller 10 kDa chaperonin, the GroES. These have been shown to be essential for cellular growth at all temperatures (Fayet *et al*., 1989; Horwich *et al*., 1993). GroEL is a tetradecamer of identical subunits arranged in two heptameric rings stacked back to back (Braig *et al*., 1994; Xu *et al*., 1997). GroEL and GroES have been shown to aid folding of non-native or partially folded proteins through ATP-dependent cycles of release and rebinding of the polypeptide in the central cavity of the chaperonin assembly (Langer *et al*., 1992; Shtilerman *et al*., 1999). This process is regulated by the interaction of GroEL with the co-chaperonin GroES.

Crystal structures of *E.coli* GroES (Hunt *et al*., 1996) as well as its homologue from *Mycobacterium leprae* (Mande *et al*., 1996) are known to atomic resolutions. GroES is a single heptameric ring of identical subunits. The overall structure of GroES resembles a dome with an orifice in its roof of ~8–12 Å. The GroES heptamer can cap either one or both ends of GroEL resulting in either an asymmetric $(GroEL_{14})$:$(GroES_7)$ (Xu *et al*., 1997) or a symmetric $(GroEL_{14})$:$(GroES_7)_2$ complex (Azem *et al*., 1994).

The GroES monomer consists of a highly conserved hydrophobic core arranged in an irregular β-barrel with a β-hairpin extending from the top, named the 'dome loop' (Taneja and Mande, 1999). Interestingly, the tip of the dome loop is dominated by a cluster of negatively charged residues. The tip of the dome loop is also the closest approach to the 7-fold symmetry axis in the chaperonin-10 (cpn-10) or GroES structures. The clustering of acidic residues, therefore, results in a very high negative potential towards the top of the GroES dome (Hunt *et al*., 1996; Mande *et al*., 1996). The concentration of acidic residues and the intense negative potential at the dome orifice may, hence, lead to an increased flexibility of the dome and possibly the instability of the oligomeric assembly. The GroES of *E.coli* has recently been proposed to have a metastable oligomeric structure leading to an increase in the diameter of the dome orifice in solution (Timchenko *et al*., 2000). In this study, we have attempted to characterize the flexibility of the dome loop by exploiting the presence of a single tryptophan residue in the dome loop of *Mycobacterium tuberculosis* cpn-10. Our results indicate that the negative charges at the dome are the likely factors responsible for the plasticity of cpn-10 structures.

Materials and methods

Multiple sequence alignment

Sequences of cpn-10 homologues were retrieved from the Swiss-Prot database (Release 36.0) (Bairoch and Apweiler, 1999). Sequence alignments were carried out using the ClustalW program (Thompson *et al*., 1994) employing the BLOSUM matrix (Henikoff and Henikoff, 1993). Default values of 10 and 0.05 for the gap opening and gap extension penalty, respectively, were used for the alignment.

Purification of cpn-10

Clones of *M.tuberculosis* cpn-10 in the pMAL-c expression vector (New England Biolabs) were available as a generous gift from Dr Vijay Mehra (Albert Einstein Institute of Medicine, New York, USA). Due to the choice of restriction sites in the multiple cloning sites, this construct consisted of seven additional residues incorporated at the N-terminus as observed from N-terminal sequencing of cpn-10 as well as from DNA sequencing of the gene. The additional residues were the same as reported in a similar cloning strategy for *M.tuberculosis* cpn-10 (Rosenkrands *et al*., 1999). These additional residues were deleted using the *Transformer* site-directed mutagenesis kit from Clontech. The deletion of the additional residues was confirmed by DNA sequencing.

The protein was purified according to the protocol previously described (Mehra *et al*., 1992). Briefly, *E.coli* JM109 containing the fusion construct (in pMAL-c) were grown to log-phase (A_{600} ≈0.4–0.5) and induced with 0.2 mM IPTG at 30°C overnight. The cells were then harvested by centrifugation. The pelleted bacteria were resuspended in 20 mM Tris–HCl (pH 8.0), 200 mM NaCl, 5mM EDTA, 1 mM PMSF and lysed by sonication. The lysed cells were then centrifuged at 9000 *g* to separate the cell debris. The fusion protein was purified from the crude extract by affinity chromatography over an amylose column. After elution from the column with 10 mM maltose, cpn-10 was cleaved from the maltose binding protein with 0.4% w/w factor Xa protease at room temperature for 12 h. The two proteins were finally separated over an anion

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CH10_ACTAC   --------MNIRPLHDKVILKREDVETKSAGGIVLTGSAATKSTRAKVLAVGPGRLLENGS-VHPMHVKVGDTVIFSDGYGV----KTEKID-GEEVLIISESDILAIVE--
CH10_ACYPS   --------MKIRPLHDRVLVKRQEVESKSAGGIVLTGSAAGKSTRGTVTAVGKGRVLDNGD-IKPLDVKVGDVVIFNEGYGA----KTEKID-NEELLILTESDILAIVE--
CH10_AGRTU   -----MTSTNFRPLHDRVVVRRVESEAKTKGGIIIPDTAKEKPQEGEIVAVGSGARDEAGK-VVALDVKVGDRVLFGQWS-----GTEVKLD-GEDSSIMKEADIMGIIG--
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CH10_ARATH   ------MMKRLIPTFNRILVQRVIQPAKTESGILLPEK-SSKLNSGKVIAVGPGSRDKDGK-LIPVSVKEGDTVLLPEYGG-----TQVKLG-ENEYHLFRDEDVLGTLHED
CH10_BACP3   ---------MLKPLGDRIVIEVVETEEKTASGIVLPDTAKEKPQEGRVVAVGAGRVLDNGQ-RIGRKSKVGDRVIFSKYAG-----TEVKYD-GKEYLILRESDILAVIR--
CH10_BACST   ---------MLKPLGDRVVIEVIETEEKTASGIVLPDTAKEKPQEGRVVAVGKGRVLDSGE-RVAPEVEVGDRIIFSKYAG-----TEVKYD-GKEYLILRESDILAVIG--
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CH10_BORPE   --------MALRPLHDRVIVKRLDNERKTASGIVIPDSAAEKPDQGEVVAVGPGKKTEDGK-ILPVDLKAGDKVLFGKYAG-----QTVKVD-GEELLVIREDEILAVIQ--
CH10_BRUAB   -----MADIKFRPLHDRVVVRRVESEAKTAGGIIIPDTAKEKPQEGEVVAAGAGARDEAGK-LVPLDVKAGDRVLFGKWS-----GTEVKIG-GEDLLIMKESDILGIVG--
CH10_BUCAP   --------MKIRPLHDRVLVKRNEAELKSAGGIVLTGSAAAKSTRGTITAVGNGRVLDNGQ-IKPLDVKVGDVVIFNEGYGA----KTEKID-NEELLILNESDILAIVE--
CH10_CAUCR   --------MKFRPLGDRVLVKRVEEETKTKGGIIIPDTAKEKPQEGEVVRSGPGARNEH-T-SSPLDVKAGDRILFGKWSGTASEGTKVKVT-S-DLLIMKESDVLGVVEA-
CH10_CHLPN   -MSDQATTLRIKPLGDRILVKREEEEATARGGIILPDTAKKKQDRAEVLVLGTGKRTDDGT-LLPFEVQVGDIILMDKYAG-----QEITID-DEEYVILQSSEIMAVLK--
CH10_CHLPS   -MSDQATTLRIKPLGDRILVKREEEDSTARGGIILPDTAKKKQDRAEVLVLGTGKRDKDGN-VLPFEVTVGDTVLIDKYAG-----QELTVD-GEEYVIVQESEVMAVLK--
CH10_CHLTR   -MSDQATTLKIKPLGDRILVKREEEASTARGGIILPDTAKKKQDRAEVLALGTGKKDDKGQ-QLPFEVQVGDIVLIDKYSG-----QELTVE-GEEYVIVQMSEVIAVLQ--
CH10_CHRVI   --------MNIRPLHDRVVVRRMEEERLSAGGIVIPDSATEKPIQGEIIAVGHGKILDNGS-VRALDVKVGDSVLFGKYSG-----TEVKLD-GKEFLVMREEDIMAVVEG-
CH10_CLOAB   --------MKIRPLGDRVVIKRLEAEETTKSGIVLPSSAKEKPQMAEVVAVGPGGVVD-GK-EIQMQVKTGDKVFFSKYSG-----TEIKVD-NEELLILRQDDILGIVEE-
CH10_CLOPE   --------MSIKPLGDRVVIKRLEAEETTKSGIIVTGTAKERPQEAEVVAVGPGAIVD-GK-RTEMEVKIGDKVLYSKYAG-----TEVKFE-GEEYTILRQDDILAIVE--
CH10_CLOTM   --------MNIRPLGDRVVVKMVETEETTKSGIVLPGSAKEKPQVAEVVAVGPGTVVD-GK-EVKMEVKVGDKVIISKYAG-----TEVKFD-GQEYTILRQNDILAVVE--
CH10_COWRU   --------MNLNMLHDNVLIEALE-ESLNNSPIQLPESAKKKPTKGKVVAVGPGSYNNNGN-LIPMTLKVGDVVFYRQWAG----NEVEFSD--KKYIVMKESDIIAKEV--
CH10_COXBU   --------MKIRPLHDRVVVRRLEEERTSAGGIVIPDSAAEKPSRGEVISVGPGKPLDNGE-VRSLDVKVGDQILFGKYAG-----TEVKLA-GDEYIVMREDDIMGVIEK-
CH10_CYAPA   MATVTLNVKTVRPLGERVLVKVSQSEEKTAGGILLPDTVKEKPQIGEIIAEGPGRRNDDGS-FQPLEVTVNSKVLYSKYAG-----TDIKLE-NEEYVLLSEKDILAIIA--
CH10_ECOLI   --------MNIRPLHDRVIVKRKEVETKSAGGIVLTGSAAAKSTRGEVLAVGNGRILENGE-VKPLDVKVGDIVIFNDGYGV----KSEKID-NEEVLIMSESDILAIVEA-
CH10_EHRCH   --------MNLNMLHDNVLIEALE-ECNSSSPIQLPDSAKKKPTQGKVVAVGPGVYNHSGN-ILPMTIKVGDVVFYRQWAG----NEIEFHE--KKYIVMKESDIIAKEA--
CH10_HAEDU   --------MSIRPLHDKVILKREEVETCSAGGIVLTGSATVKSTRGKVIAVGTGRLLENGS-IQPLSVKEGDMVIFNEGYGA----KVEKID-GEEILILSENDILAIVE--
CH10_HAEIN   --------MNIRPLHDRVIIKREEVETRSAGGIVLTGSAATKSTRAKVLAVGKGRILENGT-VQPLDVKVGDIVIFNDGYGV----KSEKID-GEEVLIISENDILAIVE--
CH10_HUMAN   ---AGQAFRKFLPLFDRVLVERSAAETVTKGGIMLPEKSQGKVLQATVVAVGSGSKGKGGE-IQPVSVKVGDKVLLPEYGG-----TKVVLD-DKDYFLFRDGDILGKYVD-
CH10_LACLA   ---------MLKPLENRVVLRVKEEEEKSMGGIVLTSASQEKPQTAEVVAVGEGKTNHHGT-LISPLVKVGDTVIFEKFAG-----TTVKMD-GEEFLILKDSDLLAIVE--
CH10_LEGMI   --------MKIRPLHDRVVVRRMEEERTTAGGIVIPDSATEKPTRGEIIAVGPGKVLENGD-VRALAVKVGDVVLFGKYSG-----TEVKIS-GQELVVMREDDIMGVIEK-
CH10_LEGPN   --------MKIRPLHDRVVVRRMEEERTTAGGIVIPDSATEKPMRGEIIAVGAGKVLENGD-VRALAVKVGDVVLFGKYSG-----TEVKVD-GKELVVMREDDIMGVIEK-
CH10_LEPIN   -------MASIKPLGDRVLVEPRQEAEEKIGSIFVPDTAKEKPQEGKVVEIGSGKYED-GK-LIPLEVKVGDTVLYGKYSG-----TEIKSE-GKEYLIIRESDILAVVKK-
CH10_MOUSE   ---AGQAFRKFLPLFDRVLVERSAAETVTKGGIMLPEKSQGKVLQATVVAVGSGGKGKSGE-IEPVSVKVGDKVLLPEYGG-----TKVVLD-DKDYFLFRDSDILGKYVD-
CH10_MYCBO   ------AKVNIKPLEDKILVQANEAETTTASGLVIPDTAKEKPQEGTVVAVGPGRWDEDGEKRIPLDVAEGDTVIYSKYGG-----TEIKYN-GEEYLILSARDVVGRRFK-
CH10_MYCLE   ------AKVKIKPLEDKILVQAGEAETMTPSGLVIPENAKEKPQEGTVVAVGPGRWDEDGAKRIPVDVSEGDIVIYSKYGG-----TEIKYN-GEEYLILSARDVLAVVSK-
CH10_MYCTU   ------AKVNIKPLEDKILVQANEAETTTASGLVIPDTAKEKPQEGTVVAVGPGRWDEDGEKRIPLDVAEGDTVIYSKYGG-----TEIKYN-GEEYLILSARDVLAVVSK-
CH10_NEIGO   --------MTIRPLHDRVVVKRLEAEEKTASGIVLPGAAAEKPDMGEVIAVGAGKIGKDGA-RRPLDVKAGDKIIFGKYSG-----QTVKAD-GEELLVMREEDIFGIVEK-
CH10_PASMU   --------MNIRPLHDRVIIKREEVETRSAGGIVLTGSAATKSTRAKVLAVGKGRILENGT-VQPLDVKVGDTVIFNDGYGV----KAEKID-GEEVLIISENDILAIVE--
CH10_PORGI   --------MNIKPLADRVLVKPAAAEEEKTVSGIIIPDSAKEKPLKGEVIAVGNGTKDEE------MVLKAGDTVLYGKYA-----GTEIELE-GEKYIIMRQNDVLAII---
CH10_PSEAE   --------MKLRPLHDRVVIRRSEEETKTAGGIVLPGSAAEKPNRGEVVAVGTGRVLDNGE-VRALAVKVGDKVVFGPYSGS----NAIKVD-GEELLVMGESEILAVLED-
CH10_PSEPU   --------MKLRPLHDRVVIRRSEEESKTAGGIVLPGSAAEKPNRGEIVAVGTGRILENGE-VRALAVKVGDKVVFGPYSGS----NTVKVD-GEDLLVMAENEILAVIEG-
CH10_RAT     ---AGQAFRKFLPLFDRVLVERSAAETVTKGGIMLPEKSQGKVLQATVVAVGSGGKGKGGE-IQPVSVKVGDKVLLPEYGG-----TKVVLD-DKDYFLFRDGDILGKYVD-
CH10_RHOCA   --------MAFKPLHDRVLVKRVQSEEKTKGGLIIPDTAKEKPAEGEVVAVGAGARKDSGE-LIAPAVAVGDRILFGKWS-----GTEVTLD-GVEMLIMKESDIMGIIS--
CH10_RICTS   --------MKYQPLYDRVLVEPIQ-NDEAHGKILIPDTAKEKPTEGIVVMVGGGYRNDKGD-ITPLKVKKGDTIVYTKWAG-----TEIKLE-SKDYVVIKESDILLVKS--
CH10_RICTY   ---------SFKPLHDRIAIKPIENEEKTKGGIIIPDTAKEKPMQGEIVAVGNGVLNKNGE-IYPLELKVGDKVLYGKWA-----GTEIEIK-GEKLIVMKESDVFGIIN--
CH10_SPIOL   ASITTSKYTSVKPLGDRVLIKTKIVEEKTTSGIFLPTAAQKKPQSGEVVAIGSGKKVG-DK-KLPVAVKTGAEVVYSKYTG-----TEIEVD-GSSHLIVKEDDIIGILET-
CH10_STAAU   ---------MLKPIGNRVIIEKKEQEQTTKSGIVLTDSAKEKSNEGVIVAVGTGRLLNDGT-RVTPEVKEGDRVVFQQYAG-----TEVKRD-NETYLVLNEEDILAVIE--
CH10_STAEP   ---------MLKPLGNRVIIEKKEQEQAAKSGIVLTDSAKEKSNEGVIIAVGQGRLLDNGT-QVAPQVSEGDTIVFQQYAG-----TEVKRG-AQTYLILNEEDILAIIE--
CH10_STRAL   -MTTASSKVAIKPLEDRIVVQPLDAEQTTASGLVIPDTAKEKPQEGVVLAVGPGRFE-NGE-RLPLDVKTGDVVLYSKYGG-----TEVKYN-GEEYLVLSARDVLAIIEK-
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CH10_SYNP6   MAAVSLSVSTVTPLGDRVFVKVAEAEEKTAGGIILPDNAKEKPQVGEIVAVGPGKRNDDGS-RQAPEVKIGDKVLYSKYAG-----TDIKLG-NDDYVLLSEKDILAVVA--
CH10_SYNP7   MAAVSLSVSTVTPLGDRVFVKVAEAEEKTAGGIILPDNAKEKPQVGEIVAVGPGKSNDDGS-RQAPEVKIGDKVLYSKYAG-----TDIKLG-NDDYVLLSEKDILAVVA--
CH10_SYNY3   -AAISINVSTVKPLGDRVFVKVSPAEEKTAGGILLPDNAKEKPQIGEVVQVGPGKRNDDGT-YSPVEVKVGDKVLYSKYAG-----TDIKLG-GDDYVLLTEKDILASVA--
CH10_THEBR   --------MRLKPLGDRVVVKVIQAEEVTKGGVILPGTAKEKPQQGEVVAVGTGEYID-GK-KVELEVKVGDRVIFSKYAG-----TEVKLD-GEEYLLLRESDILAIIE--
CH10_THETH   ---AAEVKTVIKPLGDRVVVKRIEEEPKTKGGIVLPDTAKEKPQKGKVIAVGTGRVLENGQ-RVPLEVKEGDIVVFAKYGG-----TEIEID-GEEYVILSERDLLAVLQ--
CH10_XANMA   --------MNIRXLHDRVIVKRKEVETKSAGGIVLTGSAAAKSTRGEVLAVGNGRILENGE-VKPLDVKVGDIVIFNDGYGV----KSEKID-NEEXLIMSDSDXLAIVEA-
CH10_YEAST   MSTLLKSAKSIVPLMDRVLVQRIKAQAKTASGLYLPEKNVEKLNQAEVVAVGPGFTDANGN-KVVPQVKVGDQVLIPQFGG-----STIKLGNDDEVILFRDAEILAKIAKD
CH10_YEREN   --------MKIRPLHDRVIVKRKEVESKSAGGIVLTGTAAGKSTRGEVLPVGNGRILDNGE-IKPLDVKVGDIVIFNDGYGV----KSEKID-HEEVLIMSESDILAIVEA-
CH10_ZYMMO   --------MNFRPLHDRVLVRRVAAEEKTAGGIIIPDTAKRKPQEGEVIAAGNGTHSEDGK-VVPLDVKAGDRVLFGKWS-----GTEVRVD-GEDLLIMKESDILGIIS--
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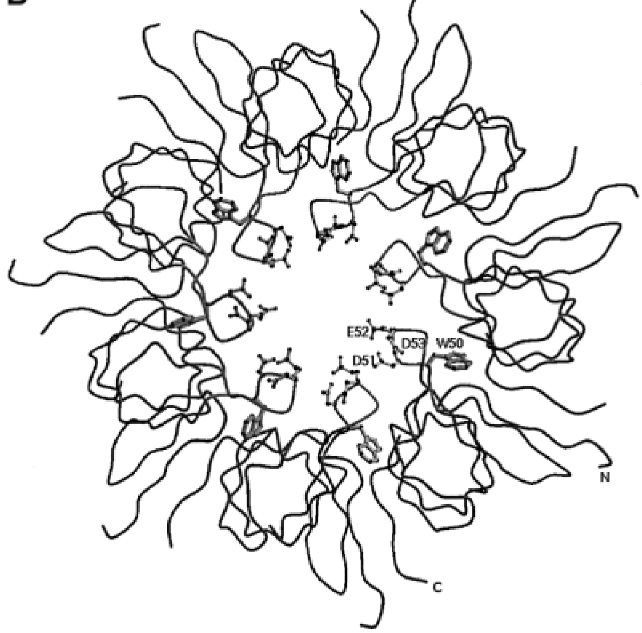

exchange column (Mono Q, Pharmacia) using fast protein liquid chromatography. The purified protein appeared as a single band on 15% SDS–PAGE (Laemmli, 1970) as detected by silver staining. The homogeneity of the protein was further confirmed over a Superdex-75 column (Pharmacia) by the elution of the protein as a single peak of 70 kDa. The concentration of the protein was determined by the method of Bradford (1976). This native cpn-10 was then pooled and dialyzed extensively against 10 mM Tris–HCl (pH 7.5) and stored at 4°C for further use.

Fluorimetric measurements

Fluorimetric measurements were performed with a Perkin-Elmer LS50B luminescence spectrometer in a 1 ml capacity quartz cuvette at 25°C using an excitation wavelength of 295 nm. The emission spectrum was recorded between 310 and 390 nm without any correction for photomultiplier sensitivity. The excitation and emission slits were set at 5 and 7.5 nm, respectively.

The concentration of protein in each scan was kept at 20 μg ml^{-1} by diluting into 20 mM Tris–HCl buffer (pH 7.5). Individual salt solutions were prepared in 20 mM Tris–HCl (pH 7.5) and added to the protein solution to a final concentration of 0.5 or 10 mM. The spectra were recorded after an equilibration of at least 1 h at 25°C. The samples were centrifuged at 10 000 *g* for 10 min before recording the spectra with the clear supernatant. Appropriate buffer baselines were subtracted in each case. Each spectrum was an average of five scans.

To confirm that the shift in λ_{max} of emission was due to the cations, each sample with 0.5 mM salt was incubated with the protein along with 20 mM EDTA for at least 1 h and the spectra were recorded again as above.

Unfolding of cpn-10 with guanidine hydrochloride

For recording the spectrum of completely unfolded cpn-10, the protein was incubated with 4 M guanidine–HCl overnight at 25°C, both in the presence and absence of cations. The guanidine concentration was determined by measurement of refractive index as described by Pace *et al*. (1989). Appropriate buffer baselines were again subtracted in each case. Each spectrum was an average of five scans.

pH titration of GroES

cpn-10 was diluted into 50 mM acetate buffer (pH 4.2) to a final concentration of 20 μg ml^{-1}. The fluorescence spectra were then recorded upon excitation at 295 nm as described above to monitor the effect of pH on the fluorescence of tryptophan.

Results

Sequence analysis

Figure 1A shows the alignment of 58 cpn-10 sequences retrieved from the Swiss-Prot database. One of the conserved features in the sequence alignment is a β-hairpin at the tip of the dome. The dome loop is formed by ~10 residues (49–59 in *M.tuberculosis* sequence numbering), with residues 51–53 at the tip of the β-hairpin. In almost all the sequences examined, at least one of the three residues in the β-hairpin is acidic although position 51 is less conserved with a hydrophobic residue in many sequences. In the *M.tuberculosis* sequence, however, all the three residues in the β-hairpin are acidic, namely aspartate-51, glutamate-52 and aspartate-53 (Figure 1B).

Conserved charged residues also occur at positions other than those mentioned in the dome loop discussed above. Examination of the three-dimensional structures indicates that these residues are distributed far apart from one another in the GroES heptamer. Absence of clustering of these residues therefore does not lead to concentration of charges in any region of the protein surface. On the other hand, since the dome loop is the closest approach to the 7-fold symmetry axes of the GroES structures, the presence of charged residues at the tip of the dome leads to a concentration of the charges in a small region. The sequestration of these negatively charged residues at the tip of the dome loop might, hence, lead to plasticity of the oligomeric structure due to repulsive electrostatic forces.

The roof β-hairpin has earlier been postulated to be metastable (Hunt *et al*., 1996) and has recently been shown to have an increased dome orifice in solution (Timchenko *et al*., 2000). Introduction of a tryptophan (Trp) residue in the dome loop would help in monitoring the plasticity of the dome loop through changes in intrinsic Trp fluorescence. Among all the cpn-10 sequences retrieved from the Swiss-Prot database, only the mycobacterial sequences contain a Trp residue in the dome loop (Figure 1A), making them ideal candidates for monitoring the plasticity of the loop. Therefore, we decided to study the plastic nature of the dome loop through the intrinsic fluorescence of this naturally occurring Trp residue in *M.tuberculosis* cpn-10.

Intrinsic Trp fluorescence of M.tuberculosis cpn-10

The fluorescence emission spectrum of the native protein due to the only Trp in its sequence shows a λ_{max} of 352 nm. This suggests that the Trp is almost fully solvent exposed. The same protein when incubated with 4 M guanidine–HCl results in a marginal decrease in fluorescence intensity and a fluorescence emission maximum at 355 nm (Figure 2). There is, thus, a minor red shift in the emission maximum when the protein is unfolded.

The crystal structure of *Mycobacterium leprae* cpn-10 (Mande *et al*., 1996) had earlier shown that the Trp-50 in an analogous position is only ~25% exposed to the solvent. The remaining part of its side chain is buried in the intersubunit interface. Solvent exposure of this Trp is, therefore, a likely consequence of the loop mobility.

Effect of metal ions on fluorescence

In the presence of metal ions, fluorescence emission spectra showed dramatic results. Table I shows the fluorescence emission maxima in the presence of an individual metal ion in each experiment. It is clear that monovalent cations have no effect on fluorescence. However, divalent cations exhibit significant shifts in the emission maximum towards lower

Fig. 1. (**A**) Sequence alignment of the cpn-10 sequences. The residues at the tip of the β-hairpin of the dome loop are shown in the shaded boxes while the conserved flanking residues are indicated by asterisks. The sequence numbering with respect to the *M.tuberculosis* GroES is indicated at the top. (**B**) Structure of the *M.leprae* cpn-10 heptamer indicating Trp-50 and the acidic residues in the dome loop. The N- and C-termini for one of the subunits are also indicated. The flexible loop that interacts with GroEL but not seen in the crystal structure is not shown. The figure was prepared using the coordinates 1LEP of *M.leprae* using MOLSCRIPT (Kraulis, 1991) and Raster3D (Merritt and Murphy, 1994).

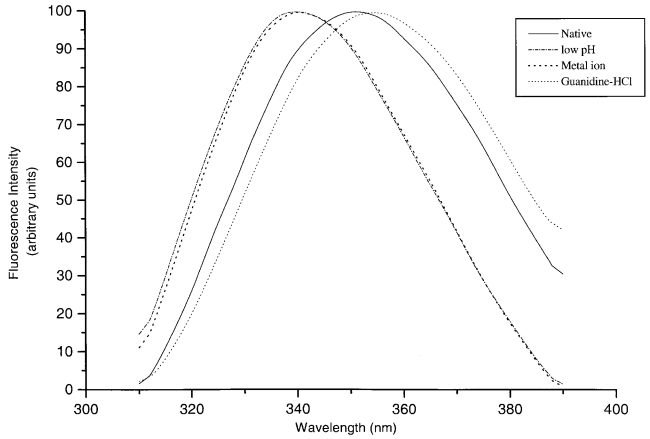

Fig. 2. Representative normalized emission spectra indicating blue shift in the presence of metal ions and at acidic pH. The emission spectrum of *M.tuberculosis* cpn-10 in the absence of metal ions is shown as a solid line. The emission spectrum in the presence of 10 mM Mn^{2+} ions (dashed line) or in the absence of metal ions at pH 4.2 (dash–dot line) shows a distinct blue shift. The red shift in λ_{max} upon unfolding in 4 M guanidine–HCl irrespective of the presence or absence of metal ions (dotted line) is also indicated. Similar results were obtained for other salts as described in the text. Addition of EDTA restored the spectrum to that of the native protein.

Table I. Effect of metal ions on the tryptophan environment of cpn-10[a]

Salt	λ_{max} (emission) (nm) (– EDTA)	λ_{max} (emission) (nm) (+ EDTA)	Change in intensity
Control (no salt)	352	352	0
Li^+	352	353	0
Na^+	353	351	0
K^+	353	353	0
Rb^+	351	353	0
Cs^+	352	352	0
Mg^{2+}	344	352	+
Ca^{2+}	344	351	++
Mn^{2+}	342	352	++
Co^{2+}	342	351	–
Ni^{2+}	342	350	++
Cu^{2+}	345	352	– –
Zn^{2+}	341	350	++
Cd^{2+}	342	352	++
Al^{3+}	342	342	+
Sm^{3+}	342	351	–
NH_4^+	352	352	0

[a]Fluorescence emission maxima upon excitation at 295 nm are indicated in the absence (– EDTA) or presence (+ EDTA) of EDTA. The results are indicated for 0.5 mM salt. Similar results were obtained for protein with 10 mM salt except for much larger changes in intensity. A change in fluorescence intensity of <10% is indicated as 0, while +, ++ and –, – – indicate an increase or decrease in fluorescence intensity of up to 50% or >50%, respectively. No change in λ_{max} is observed in the case of Al^{3+} upon addition of EDTA, due to the probable dual effect of decrease in pH upon addition of aluminium chloride in water.

wavelengths (Figure 2). The shift in λ_{max} in the presence of divalent cations suggests stabilization of the dome loop with a concomitant burial of the Trp into a different metastable state of cpn-10.

In order to confirm if the effect of the blue shift in λ_{max} of fluorescence emission was indeed due to the cations, fluorescence spectra of the protein were recorded in the presence of EDTA. Due to the 40-fold molar excess of EDTA over metal ions in solution, EDTA was anticipated to chelate the metal ions and thereby reduce their interaction, if any, with cpn-10. The results listed in Table I clearly show that in the presence of EDTA, the λ_{max} appears in the same position as the native protein, with a clear shift from the corresponding position as observed in the absence of EDTA. Upon addition of EDTA, the fluorescence intensity was also restored to that of the native protein.

Effect of pH titration on fluorescence

The fluorescence spectrum of the *M.tuberculosis* cpn-10 in 50 mM acetate buffer (pH 4.2) recorded in the absence of cations yields a fluorescence emission maximum at 341 nm. This amounts to a blue shift of ~11 nm as compared to the native protein in Tris buffer (pH 7.5) (Figure 2). At pH 4.2, the dome carboxylates are expected to be protonated, losing their negative charge. The unionized states of the carboxylic groups are thus expected to result in a decrease in the negative potential at the top of the cpn-10. Consequently, there would be decreased repulsions between the β-hairpins of the dome leading to the burial of the tryptophans in the hydrophobic interface.

Discussion

The cpn-10 structures are known to be highly plastic in nature. Crystal structures of two cpn-10s had earlier revealed significant deviations from a perfect 7-fold symmetry (Hunt *et al*., 1996; Mande *et al*., 1996), and therefore had indicated the delicate nature of their oligomeric assembly. The plasticity of the heptameric assembly partly arises from the dome loop, which forms a part of the monomer–monomer interface, and is believed to be flexible in solution (Timchenko *et al*., 2000). The tip of the dome loop possesses invariant acidic residues (Figures 1A and B). The presence of these acidic residues at the tip of the loop is believed to lead to an intense negative potential at the dome oculus, and perhaps to the destabilization of the oligomeric assembly. The dome loop is flanked by two highly conserved stretches of residues, a short glycine-rich stretch (positions 46–48) preceding the loop and an invariant glycyl-aspartyl dipeptide at positions 65 and 66, following the loop (Taneja and Mande, 1999). One of the glycine residues preceding the dome loop at position 48 of *M.tuberculosis* GroES has a positive phi dihedral angle in the *M.leprae* as well as *E.coli* cpn-10 structures. Its conservation in evolution might therefore be important for structural reasons. Similarly, the succeeding stretch of the highly conserved glycyl-aspartyl dipeptide at positions 65 and 66 has earlier been shown to be responsible for the maintenance of the GroES-fold (Taneja and Mande, 1999). The evolutionary conservation of flanking residues on either side of the loop, as well as that of the charged residues at its tip, suggest a possible critical role of the dome loop in cpn-10 function.

We have attempted to study the conformational plasticity of cpn-10 by taking advantage of a single Trp residue in the dome loop of *M.tuberculosis* cpn-10. The Trp residue, which is present within the loop, behaves as if it is fully solvent exposed in the native structure. The emission maximum of its fluorescence shifts very little towards higher wavelength under fully denaturing conditions. There is, however, a dramatic shift in λ_{max} of the Trp in the presence of divalent cations. The blue shift of ~8–11 nm in the presence of divalent cations is indicative of solvent shielding of the Trp as seen in the crystal structure of cpn-10. This result is further corroborated by restoration of λ_{max}, as that in the native protein, in the presence of EDTA along with the divalent cations. The divalent cations

therefore serve to stabilize the negative charges at the tip of the dome loop.

Additional evidence for the loss in flexibility is provided by blue shift in λ_{max} of Trp at lower pH. Thus, when the acidic residues lose their charge, the environment of the Trp residue in the dome loop becomes less polar. The shift in Trp fluorescence in the presence of divalent cations, its restoration in the presence of EDTA, and a similar blue shift at acidic pH indicate that the plasticity of the dome loop is due to the negatively charged residues at its tip.

Escherichia coli GroES has recently been shown to have an increased dome orifice in solution than seen in its crystal structure (Timchenko *et al*., 2000). While the orifice of the dome is only 8 Å wide in the crystal structure, it opens by as much as 10–15 Å in solution. This may be a consequence of the intrinsic flexibility of the dome loop due to the negative charges as seen for *M.tuberculosis* GroES in this study. Although the significance of dome loop dynamics is as yet unclear, we believe that our demonstration of its flexibility should lead to a better understanding of its function. It has also been previously suggested that stoichiometric binding of Mg^{2+} stabilizes the *E.coli* GroES protein (Boudker *et al*., 1997). A number of divalent cations have also been previously reported for the maintenance of the proper oligomeric state of *M.tuberculosis* GroES (Fossati *et al*., 1995). In this report, we have shown that the plasticity of cpn-10 is due to the high negative potential at the orifice of the dome and is modulated by various metal ions.

It is interesting to note that the chelation of magnesium ions by EDTA or CDTA (*trans*-1,2-diaminocyclohexane *N*,*N*,*N*′,*N*′-tetraacetic acid) disrupts the GroES:GroEL association (Hayer-Hartl *et al*., 1995; Feltham and Gierasch, 2000). One of the mechanisms for this disruption is the depletion of nucleotides from the GroEL pool. This results in the 'open' conformation of GroEL as in the trans ring (Xu *et al*., 1997) or as in the stand-alone structure that cannot bind GroES. However, it is plausible that simultaneous conformational changes in GroES in the absence of divalent cations further reduce its affinity for GroEL. The dome loop might hence utilize the negative charges at its tip and their neutralization with metal ions to act as a 'conformational switch' for interaction with GroEL.

Acknowledgements

We thank Vijay Mehra for the gift of *M.tuberculosis* cpn-10 clones. We also thank Girish Sahni and Paramjit Kaur for help in N-terminal sequencing, Avinash Kale for help in fluorescence experiments, and B. Gopalakrishnan for useful suggestions and critical reading of the manuscript. IMTECH and Department of Biotechnology provided financial support for the work. B.T. is a CSIR senior research fellow.

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Received October 17, 2000; revised February 22, 2001; accepted March 2, 2001