Structure and stability of the C-terminal helical bundle of the *E. coli* mechanosensitive channel of large conductance

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Abstract: The crystal structure of the cytoplasmic domain (CTD) from the mechanosensitive channel of large conductance (MscL) in *E. coli* has been determined at 1.45 Å resolution. This domain forms a pentameric coiled coil similar to that observed in the structure of MscL from *M. tuberculosis* and also found in the cartilage oligomeric matrix protein (COMPcc). It contains canonical hydrophobic and atypical ionic interactions compared to previously characterized coiled coil structures. Thermodynamic analysis indicates that while the free EcMscL-CTD is less stable than other coiled coils, it is likely to remain folded in context of the full-length channel.

Keywords: protein structure; mechanosensitive channels; membrane proteins; coiled coils

Introduction

Bacteria must respond to a variety of environmental stimuli including osmotic stress. Changes in environment that lead to hypo-osmotic shock cause an influx of water into the cell resulting in significant swelling of the cell. Since the inner membrane has limited elasticity, proteins embedded in this membrane are essential to protect the cell from membrane damage, particularly lysis under these conditions.¹ Mechanosensitive channels, including the mechanosensitive channel of large conductance (MscL), serve this protective function by opening large, nonselective pores, thereby allowing solutes and water to equilibrate across the membrane in response to hypo-osmotic shock.^{2–4}

The mechanism of MscL has been studied by functional and biophysical approaches since the identification of the *mscL* gene by Kung and coworkers.⁵ MscL is an intrinsically stretch activated channel, which responds to an increase in membrane tension due to hypo-osmotic stress by opening to form a pore of ~30 Å diameter.^{6–10} Given the small size of the MscL subunit (136 residues for *E. coli* MscL (EcMscL)), it must oligomerize to form a pore capable of producing such a large opening and consequently a large measured conductance.⁵ This oligomerization has been confirmed using several methods including crosslinking,^{11,12} crystallography¹³ and the oligomer characterization by addition of mass (OCAM) method.¹⁴ The structure of *Mycobacterium tuberculosis* MscL (MtMscL) has been solved in a nonconducting state as a homopentamer,^{13,15} while a C-terminal truncation of *Staphylococcus aureus* MscL (SaMscL) has been solved in an expanded, nonconducting conformation as a homotetramer.¹⁶ Despite the oligomeric state variability observed for overexpressed and detergent solubilized MscLs,^{14,17} the pentamer likely predominates in the membrane under normal expression levels.¹⁸

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Figure 1. Structure of EcMscL-CTD. A: Ribbon diagram of MtMscL (PDB code: 2OAR) with four of five chains in transparent gray and the fifth chain colored according to structural domain. N-terminal helix in orange, TM1 in purple, periplasmic loop in red, TM2 in green, and C-terminal domain in yellow. B: Superposition of EcMscL-CTD (residues E108–S136) in color onto MtMscL-CTD in gray (residues E102–N125). C: Ribbon diagram of EcMscL-CTD viewed down the pseudo-5-fold symmetry axis from the N-terminus. The loop region is defined between the labeled residues E108 and T115. D: Alignment of EcMscL-CTD with MtMscL-CTD. Heptad repeats are labeled above the alignment with **a**, **d**, **e**, and **g** positions in bolded blue type. Similar amino acids are in light orange and identical amino acids are in green. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The underlying determinants of MscL oligomeric state must reflect interactions between the constituent subunits. The MscL subunit contains two transmembrane helices^{5,13,16} [TM1 and TM2; Fig. 1(A), indicated purple and green], connected by a periplasmic loop [Fig. 1(A), red]. The TM1 helices pack together around the symmetry axis that coincides with the permeation pathway. The packing of TM1 helices allows for changes in helix tilt during the gating transition,⁸ thus making multiple oligomeric states compatible with the same helix–helix interface,¹⁹ and also making it unlikely that TM1–TM1 packing interactions uniquely specify the oligomeric state of MscL.¹⁴ Of the nonmembrane spanning components of the MscL subunit, there is an essential²⁰ cytoplasmic N-terminal helix [Fig. 1(A), orange] oriented parallel to the membrane plane, a periplasmic loop, and a cytoplasmic C-terminal domain [CTD; Fig. 1(A), yellow]. The dominant feature of the CTD in the MtMscL structure is the presence of an α -helical bundle organized as a pentameric coiled coil. The hydrophobic residues comprising the helix–helix interface of the CTD coiled coil are among the more highly conserved residues in MscL (see Hidden Markov Model (HMM) Logo, <http://pfam.janelia.org/family/PF01741>), which supports a conserved role for this region in maintaining MscL structure and function. Given the extensive intersubunit interactions present in the cytoplasmic domain (CTD), it is reasonable to expect this element to influence the oligomeric state of MscL.

The possibility that this region is crucial for the proper oligomeric assembly of MscL has been studied using our OCAM method, where we find that a C-terminal truncation of MtMscL remains pentameric as observed for the full length protein. In contrast, the full length EcMscL appears to be a mixture of pentamers and hexamers, and while full length SaMscL is a pentamer, truncated SaMscL can form both pentamers and tetramers.¹⁴ When measured using crosslinking, the membrane inserted, rather than detergent solubilized SaMscL (full length and CTD truncation) appears pentameric.^{17,21} These data indicate that while the cytoplasmic bundle influences the observed variability of MscL oligomeric state, this domain is not the sole determinant.

Achieving the estimated 30 Å diameter pore^{6–10} of the MscL open state would seemingly require dissociation of the CTD coiled coil. This consequence was suggested in the original MtMscL structure determination¹³ and was more fully developed in the studies of Sukharev and Guy.^{8,22} Electron microscopy (EM) experiments on a constitutively open channel mutant (G22N) showed a loss of electron density for the cytoplasmic bundle compared to wild-type protein, interpreted as indicating dissociation of the CTD when the channel is in an open conformation.²³ Passage of ~20 Å protein molecules through the MscL pore further indicates dissociation of the CTD upon channel opening.¹⁰ However, subsequent electrophysiological measurements of MscL when

the CTD is crosslinked via engineered disulfide bonds indicates that ions can still pass through the pore without complete dissociation of the coiled coil.²⁴ More recently it has been shown that reduction of the linker length between TM2 and the CTD leads to a reduced conductance through the pore as well as increased MscL gating pressure.²⁵ These results along with further CTD crosslinking in the same study indicate that the CTD does not dissociate upon channel gating.²⁵

The inability to precisely define the role of the CTD mirrors the results of random screens indicating that MscL function is largely insensitive to mutagenesis of this region.^{26,27} Furthermore, forms of EcMscL with the C-terminal helix truncated are functional both *in vivo* and *in vitro*,^{20,24} again indicating that this region is not essential for MscL gating; similar observations were subsequently made on the SaMscL homolog.¹⁶ The inconsistency between sequence conservation, yet apparent dispensability of the CTD has not been resolved and may reflect our lack of detailed understanding of the MscL gating mechanism. To address these questions, structural and biophysical studies of the isolated EcMscL CTD were undertaken to provide insight into the role of this region in influencing the oligomeric state and function of MscL.

Results

A structure of the EcMscL-cytoplasmic domain (EcMscL-CTD), consisting of residues 108–136, was determined using X-ray crystallography to 1.45 Å resolution [Fig. 1(B,C) and Supporting Information Table 1]. Although EcMscL is the best functionally characterized MscL homolog, structural characterization of the full-length channel has been problematic and this structure represents the first crystallographic characterization reported for this system. The 29 residue domain is divided into two structural regions [Fig. 1(B,C)], a pentameric α -helical coiled coil (Thr116–Ser136) and an irregular, more extended region (Glu108–Pro115). The coiled-coil closely resembles the corresponding structure in MtMscL [Fig. 1(B)], and the irregular region (which is fully defined for only two of the five subunits) also exhibits an overall conformation similar to that seen connecting TM2 and the helical bundle in MtMscL [Fig. 1(A)].

The EcMscL-CTD Contains a Canonical Pentameric Coiled Coil

Coiled coils consist of two or more α -helices wrapped together to form twisted rope-like structures.²⁸ Coiled coils are defined by a heptad repeat in primary sequence represented as **abcdefg**, where hydrophobic residues such as isoleucine and leucine occupy the **a** and **d** positions, especially in dimeric coiled coils,^{29,30} and produce a surface along one face of each helix that allows a “knob-into-hole” packing

arrangement between an **a** residue “knob” and a **dgad** “hole.”³¹ This packing arrangement is often referred to as KIH (knob in hole) packing. Naturally occurring coiled coils have been observed in several different oligomeric forms, from dimer up to pentamer.^{32,33} In addition to the interactions of residues at positions **a** and **d** in the heptad repeat, pentameric coiled coils also have interactions between the residues at positions **e** and **g** due to the increased burial of these residues in higher order oligomeric coiled coils, as exemplified by cartilage oligomeric matrix protein (COMP³⁴). The identity of residues in the **e** and **g** positions is more variable than positions **a** and **d**. Typically, knobs are formed by **a**, **d**, **e**, and **g** residues and pack into holes formed between **a'–g'**, **e'–d'**, **c'–d'**, and **a'–b'**, respectively (primes denote residues on adjacent chain [Fig. 2(C)]).^{28,32}

The EcMscL-CTD contains nearly three heptad repeats starting with residue Thr116 [**b** in the heptad; Fig. 1(D)]. The hydrophobic interactions in the heptad repeat occur in the highly conserved region of the bundle between residues Leu122, Ile125, and Leu129 which occupy **a**, **d**, and **a**, positions in the heptad respectively. The corresponding **g** and **e** positions in this region are residues Leu121 (**g**), Arg126 (**e**), and Leu128 (**g**). Two examples of KIH packing can be seen in Figure 2, where Leu122 (**a**) packs between Leu121 (**g'**) and Leu129 (**a'**) [Fig. 2(A)] and Leu128 (**g**) packs between Leu129 (**a'**) and Lys130 (**b'**) [Fig. 2(B)]. A schematic helical wheel diagram in Figure 2(C) illustrates these packing interactions for all three heptad repeats present in the EcMscL-CTD. These packing interactions are typical of those commonly observed in coiled coils.

An informative comparison may be made between the EcMscL-CTD and the pentameric coiled coil of COMP (COMPcc³⁴). COMPcc is one of a small number of naturally occurring pentameric coiled coil domains, and consists of 46 residues, over twice the length of the 20 residue EcMscL-CTD coiled coil. Despite this length difference, EcMscL-CTD is structurally similar to COMP (PDB code: 1VDF), as reflected in the 0.46 Å rmsd when α -carbons from chain D are fit to the α -carbons of chain C in COMP [Fig. 3(A,B)]. This similarity is also evident in the matching pattern of hydrophobic and polar residues at the core of EcMscL-CTD and COMPcc [Fig. 3(C)] where three heptad repeats are superimposed. The **a** positions in Heptads 2 and 3 are identical, while the **d** positions are similar for Heptads 1 and 2, and identical in the third. Positions **e** and **g** are similar in Heptad 1, and identical in Heptad 2, while in Heptad 3, position **g** is similar [Fig. 3(C)]. Also present in EcMscL-CTD is a water molecule coordinated by four Gln132 side chains in a similar position to the chloride ion coordinated by all five Gln54 side chains in COMPcc. Since both of these domains assemble into pentameric coiled coils, these similarities are

expected and may further elucidate the sequence determinants for pentameric coiled coils.

Networks of electrostatic interactions are found at the top, middle, and bottom of the EcMscL-CTD F4 [Fig. 4(A)]. Residue Glu118 occupies the first **d** posi-

tion, but instead of packing in a typical KIH fashion between **e'** and **d'** on the neighboring helix, four of the five glutamate side chains in this position coordinate two water molecules [Fig. 4(B)]. These interactions form an electrostatic "cap" at the top of the helical bundle, near the membrane proximal region of the EcMscL-CTD. Residue Arg126, which occupies the **e** position in the central heptad, is involved in a network of salt bridges with Glu124 (heptad position **c'**) on the neighboring helix. These interactions extend around the circumference of the bundle forming a central electrostatic "belt" around the middle of the coiled coil [Fig. 4(C)]. Arg126 is also held into position by salt bridges to Asp127 (heptad position **f**) on the same helix. The C-terminal end of the bundle is stabilized by hydrogen bonds between Arg135 and Ser136 (involving the side chain hydroxyl or C-terminal carboxyl group) on the adjacent helix, which form a hydrogen bond network around the cytoplasmic end of the EcMscL-CTD [Fig. 4(D)].

These H-bond networks at either end of the EcMscL-CTD coiled coil are also reminiscent of similar interactions observed in COMPcc. The electrostatic belt observed in EcMscL-CTD is not present in the COMPcc structure, however, it could potentially form through interactions between Arg48, Asp46, and Glu49. In fact, salt bridges are observed between Arg48 and Asp46 on the neighboring helix in two of the five positions of COMPcc, but the network does not extend around circumference of COMPcc as it does in EcMscL-CTD. The engineered pentameric coiled coil based on the *E. coli* outer membrane lipoprotein Lpp (Phe-14) also contains an arginine residue (Arg31) with the capability to form this electrostatic belt.³⁵ Indeed salt bridges are observed between Arg31 and Asp33 on the neighboring helix in four of the five helices in the Phe-14 coiled coil. In the fifth case Arg31 is in proximity to Asp26 on the adjacent helix (PDB code: 2GUV).

Figure 2.

Figure 2. Knob-into-hole packing in EcMscL-CTD. A: Knob-into-hole packing of L121 (magenta) in position **g** and L122 (cyan) in position **a**. Note that L121 only participates as part of a "hole" while L122 acts in both types of interactions as described by an **a** knob packing into an **a'/g'** hole. The C α backbone trace is in green. B: Knob-into-hole packing of L128 knob (orange) in position **g** into hole formed by L129 (cyan) in position **a'** and K130 (magenta) in position **b'**. L129 also acts as a knob (**a**) packing in the hole formed by L129 (**a'**) and L128 (**g'**). C: Helical wheel diagram of three heptad repeats in EcMscL-CTD (residues T116–Q135). Hydrophobic residues are colored gray, polar residues are orange, basic residues are blue, and acidic residues are red. <http://imolecules3d.wiley.com:8080/imolecules3d/review/HcJu3zEwUjW7AfHE517mXLg1fTZzNU5PVaOBXurRPYdHY0W5U9gLLSHjhONYTGrP720/1375> [An interactive view is available in the electronic version of the article.] [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 3. Comparison of EcMscL-CTD with COMPcc. A: Side view of superposition of EcMscL-CTD α -carbon trace in green onto COMPcc α -carbon trace in magenta with side chains of identical residues shown as sticks. B: The view of (A) along 5-fold symmetry axis looking toward the C-terminus for visualization of the internal cavity created by the coiled coil. C: Sequence alignment of EcMscL-CTD with COMPcc. Heptad repeats are labeled above the alignment with **a**, **d**, **e**, and **g** positions in bolded blue type. Similar amino acids in these positions are in light orange and identical amino acids are in green. Note that only position **e** in the third heptad repeat is not similar or identical for these important interactions in pentameric coiled coils. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 4. Electrostatic interactions in EcMscL-CTD. A: EcMscL-CTD electrostatic interactions at top, middle, and bottom of the helical bundle. A detailed view of each interaction is shown in panels (B–D). B: Top: Electrostatic cap (membrane proximal) created by H-bond network between E118 and two water molecules. C: Middle: Electrostatic belt created by salt bridge between R126 and E124 on adjacent helix. D: Bottom: Electrostatic cap (membrane distal) created by H-bond network between R135 and C-terminal S136 on adjacent helix. <http://imolecules3d.wiley.com:8080/imolecules3d/review/HcJu3zEwUjW7AfHE517mXLg1fTZzNU5PVaOBXurRPYdHYOW5U9gLLSHjhONYTGrP720/1377> [An interactive view is available in the electronic version of the article.] [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 5. Thermodynamic analysis of EcMscL-CTD unfolding. A: Melting analysis of EcMscL-CTD. Plot of data and theoretical fits to the data from least squares analysis. Colors of spectrum correspond to low concentration (28 μM) in red up to high concentration (168 μM) in pink. Inset: theoretical melting curves for 10 mM (black), 58 mM (gray), and 100 mM (light gray) peptide. The vertical line at 310 K designating physiological temperature is well below the predicted melting temperature at these concentrations. B: GdnHCl denaturation of EcMscL-CTD. ΔG_D versus GdnHCl concentration. y intercept at 108.4 kJ mol^{-1} . [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The initial model of MtMscL (PDB code: 1MSL) had registry errors that positioned charged side chains in the interior of the helix bundle and hence called into question the relevance of this structure under physiological conditions. A model for the EcMscL-CTD produced by Anishkin *et al.* based on COMPc is in excellent agreement with the structure presented here.²⁴ Subsequent to the work of Anishkin *et al.*, we re-refined the MtMscL model and corrected these registry errors to generate the current coordinate set (PDB code: 2OAR¹⁵). While the $\text{C}\alpha$ positions are in good agreement for the coiled coil regions of MtMscL and EcMscL-CTD (rms 0.38 Å), different sidechain rotamers are often observed in MtMscL relative to EcMscL-CTD; in view of the superior diffraction quality of the latter, these sidechain conformations are much more likely to be correct.

Stability of EcMscL-CTD

To address the issue of CTD dissociation during gating, we determined the thermodynamic stability of the isolated domain using circular dichroism (CD) spectroscopic measurements. By monitoring the loss of α -helical secondary structure under denaturing conditions, the thermodynamics of unfolding for the EcMscL-CTD peptide were determined, based on the assumption that α -helical character is observed only in the coiled coil.³⁶ Denaturing conditions of increasing temperature or guanidine hydrochloride (GdnHCl) were used. Melting curves were measured at various concentrations of peptide, and these data were then analyzed to determine the thermodynamic parameters for folding and unfolding. Increasing

and decreasing temperature ramps show identical profiles indicating that this process is reversible for this peptide (Supporting Information Figure 1). Data were analyzed assuming a two-state transition given by the following equation^{37–40}:

$$F_5 = 5U$$

where F_5 is a folded pentamer and U is an unfolded monomer. An optimization was done for the pentameric EcMscL-CTD with Mathematica® as described in the methods. The following values were obtained [Fig. 5(A)]. $K_0 = 4.8 \times 10^{-19}$ at $T_0 = 298$ K; $\Delta H = 254$ kJ mol^{-1} and a value of 105 kJ mol^{-1} for ΔG can be calculated from K_0 . GdnHCl denaturation was monitored at 25°C and 56 μM peptide using the CD signal at 222 nm. At 25°C and 0M GdnHCl, the peptide is ~65% folded, and it becomes fully denatured with the addition of only 1M GdnHCl. The linear extrapolation method was used to calculate $\Delta G_D^{\text{H}_2\text{O}}$ from the equation

$$\Delta G_D = \Delta G_D^{\text{H}_2\text{O}} - m[\text{denaturant}]$$

where ΔG_D was determined from Eq. (M4) in the Methods for K .³⁷ Fraction folded (f_N) was calculated using Eq. (M2) and $\theta_N = -32,600$ $\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ and $\theta_D = -9700$ $\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ obtained from the fit calculated for melting temperature ramps. This results in a value of 108 kJ mol^{-1} [Fig. 5(B)] for denaturation with GdnHCl which is in agreement with the melting temperature experiment. The value of ~100 kJ mol^{-1} for the unfolding of this peptide

represents the thermodynamic stability at the standard state of 1M peptide.

Discussion

Osmotic down-shock in bacteria causes membrane stress that is relieved by mechanosensitive channels.⁴¹ MscL is an ~120–150 amino acid oligomeric channel (depending on the homolog) that consists of an N-terminal cytoplasmic helix, two membrane-spanning helices that flank a periplasmic loop and a cytoplasmic coiled coil domain.^{5,13} Here, we show that the CTD of EcMscL crystallizes as a pentameric coiled coil as observed in the crystal structure of MtMscL^{13,15} and predicted by modeling studies carried out by Anishkin *et al.*²⁴ This coiled coil has the canonical heptad repeat found in natural coiled coils and the interactions between heptad positions **a**, **d**, **e**, and **g** fit with predictions and observations from other pentameric coiled coils, both natural and designed.^{34,35,42,43} The similarity to COMPcc is particularly striking. There are exceptions to the expected interactions, however, as seen in the electrostatic belt formed by Arg126 and Asp124 around the middle portion of the coiled coil domain. Arg126 occupies an **e** position in the heptad repeat and would be expected to pack between **c'** and **d'** but instead forms a network of salt-bridges with the **f'** positioned Asp124. This set of interactions is not unprecedented in pentameric coiled coils since similar interactions are present in Phe-14, the designed pentameric coiled coil based on Lpp-56.³⁵

In our analysis of the folding transition for this peptide, we have assumed a two state model involving a fully folded pentamer and an unfolded monomer. The data are effectively modeled with this assumption using two different denaturants (temperature and GdnHCl). When comparing the EcMscL-CTD coiled coil stability to other coiled coil systems found in the literature, it appears to be less stable than most other systems with regards to the melting temperature. By comparing the T_m values of similar systems such as COMPcc ($T_m = 41$ – 60°C under different experimental conditions at concentrations up to $10\ \mu\text{M}$ ^{44,46}) or the tetrameric variant of cartilage matrix protein (CMP) ($T_m = 58^\circ\text{C}$ at $50\ \mu\text{M}$ ^{42,45}), it is clear that EcMscL-CTD ($T_m = 29^\circ\text{C}$ at $56\ \mu\text{M}$) is less stable in solution than these other coiled coil systems (Supporting Information Table 2). This could simply be due to the short, 21 aa, nature of the EcMscL-CTD coiled coil, which has fewer stabilizing interactions than the longer coiled coils mentioned (CMP and COMPcc have 36 and 63 residues, respectively). It is also possible that the electrostatic interactions observed in EcMscL-CTD destabilize the coiled coil. This has been observed in COMPcc where mutation of polar residues T40 and Q54 to alanine result in an increased T_m for these variants and in dimeric leucine zippers where salt bridges appear to be destabi-

lizing.^{45,47} These interactions are more easily disrupted by GdnHCl than are hydrophobic interactions, which can lead to overestimation of the ΔG° when extrapolating to zero denaturant concentration,^{39,48} which may explain our relatively high ΔG° values compared to other systems that are more stable based on melting temperature analysis. There is also variability in the data treatments for GdnHCl denaturation of coiled coils reported in the literature^{39,42,44,46} (Supporting Information Table 2 and Supporting Information Fig. 2).

Extrapolation from the measured stability of EcMscL-CTD in solution under micromolar concentrations to that in the intact channel requires an estimate of the effective concentration of the CTD in MscL. The effective volume can be estimated if we use the dimensions observed in the crystal structure to approximate the volume occupied by the CTD. Assuming five molecules occupying a hemisphere (due to proximity to the membrane) with a radius of 45 Å, an effective concentration of 58 mM is calculated. At this concentration we would expect the EcMscL-CTD to essentially be in the completely folded state at a physiologically relevant temperature of 37°C [Fig. 5(A), inset]. Alternatively we can calculate the concentration of peptide when it is at least 99% folded as 5.5 mM, using Eq. (M4) and the measured value for K_0 (4.8×10^{-19}). Since this number is within an order of magnitude of the value above for effective concentration (58 mM), it becomes clear that the EcMscL-CTD is likely to be in the pentameric coiled coil state when in the context of the full-length channel. By calculating theoretical melting curves from the thermodynamic model generated from our data, at concentrations of 10, 58, and 100 mM [Fig. 5(A), inset] it also appears that the EcMscL-CTD is likely to be in the folded state when present at the expected concentration found in a properly folded and membrane embedded channel.

The structure of the isolated EcMscL-CTD solved at 1.45 Å resolution establishes that this region forms a pentameric coiled coil that closely resembles the corresponding region in the intact MtMscL structure and that this structure can form in both the context of the full length channel as well as the free peptide crystallized here. The conserved nature of hydrophobic residues forming helix–helix interfaces indicates that these interactions will be broadly relevant to the MscL family. Furthermore, the thermodynamic stability of isolated EcMscL-CTD is sufficiently high to expect the pentameric coiled-coil to remain intact during the gating transition of MscL. Yet to be explored are the consequences of sequence variation in the coiled-coil interface on oligomeric state. Deletion of the MscL CTD does have functional consequences in electrophysiological assays, but is tolerated *in vivo* as measured by

response to hypo-osmotic shock.^{16,20,24} Notwithstanding the detailed structural and biophysical characterizations, it remains problematic to reconcile the conserved sequence elements with the minimal consequences *in vivo* for mutating or deleting the C-terminal domain of MscL, suggesting perhaps there remain unrecognized selective pressures maintaining conservation of this region.

Methods

Crystallization and Data Processing

Peptides were synthesized by GL Biochem (Shanghai, China) using Fmoc chemistry. Initial crystallization screens were performed in the Caltech Molecular Observatory using commercially available sparse matrix screens and sitting drop vapor diffusion with a drop size of 100 nL peptide solution plus 100 nL well solution. Peptides were screened at 10 mg/mL dry weight in 20 mM Tris pH 7.5, 150 mM NaCl. The initial hit (Hampton Crystal Screen HT position F1) was 0.1M sodium acetate pH 4.6, 0.2M ammonium sulfate, and 30% polyethylene glycol 2000 monomethyl ether (PEG 2000 MME) at 4°C. This was optimized in 2 μ L + 2 μ L hanging drop experiments to 0.1M sodium acetate pH 4.6, 0.34M ammonium acetate, and 34% PEG 2000 MME. Crystals were cryoprotected by soaking in mother liquor plus 5% ethylene glycol for ~1 min, followed by mother liquor plus 10% ethylene glycol for an additional minute and flash frozen in liquid N₂. Data were collected at Stanford Synchrotron Radiation Lightsource (SSRL) beamline 12-2. Data were indexed and scaled using Mosflm and Scala as implemented in CCP4^{49,50} and phases were determined using molecular replacement as implemented in Phaser.^{51,52} The structure of MtMscL-CTD (residues 102–125 from PDB code: 2OAR) was used as a search model. The initial EcMscL-CTD model was built with Phenix⁵³ auto-build and iterative rounds of model building and refinement were done using COOT⁵⁴ and Phenix.

CD Spectroscopy

The EcMscL-CTD peptide stock solution was made in water at 1.12 mM and stored at –20°C. Stock concentration was determined via amino acid analysis by the Molecular Structure Facility at UC Davis. The information from this analysis was combined with UV absorbance measurements to calculate extinction coefficients at various wavelengths. For EcMscL-CTD $\epsilon_{220} = 36,747$ M^{–1} cm^{–1}. Samples for CD experiments were diluted from the 1.12 mM stock into 20 mM sodium phosphate pH 7.0, 300 mM NaCl. Data were collected on an AVIV model 62A DS CD spectrometer. Wavelength scans from 200 to 250 nm were run in triplicate with a 10 s averaging time at 1 nm increments and a 1.5 nm bandwidth. Temperature and denaturant concentrations are indicated in figure legends. Temperature ramp experi-

ments measured ellipticity at 222 nm in 2°C increments after 1 min equilibration at each temperature. Acquisition time was 10 s at each temperature. Samples in guanidine HCl were allowed to equilibrate overnight at 4°C before measurements were taken after 10-min incubation at 25°C.

Thermodynamic Analysis of CD Data

Data were analyzed assuming a two state transition of the type used in the analysis of comparable systems^{37–40}:

$$F_5 = 5U$$

where F_5 is the folded pentameric state and U is the unfolded monomeric state.

From this, the following relationships may be derived

$$C = 5[F_5] + [U] \quad (1)$$

$$f_N = (\theta - \theta_D) / (\theta_N - \theta_D) \quad (2)$$

$$[U] = (1 - f_N)C; [F_5] = f_N C / 5 \quad (3)$$

where C = total peptide concentration (M); f_N is the fraction of peptide monomer in the folded conformation; and θ_N , θ_D are the measured CD ellipticities of the two states. The equilibrium constant may then be written in terms of C and f_N :

$$K = [U]^5 / [F_5] = ((1 - f_N)C)^5 / (f_N C / 5) \quad (4)$$

$$= 5C^4 (1 - f_N)^5 / f_N \quad (5)$$

The temperature dependence of K is related to ΔH , the enthalpy change for the unfolding/dissociation reaction; when ΔH is constant (independent of T) this becomes:

$$K(T) = K_0 \exp [-(\Delta H/R)((1/T) - (1/T_0))] \quad (6)$$

where K_0 is the value of K at the reference temperature T_0 . For given values of C and K , the fraction of peptide in the folded state, f_N , can be found by solving the polynomial equation:

$$(f_N / (1 - f_N))^5 = 5C^4 / K(T) \equiv x \quad (7)$$

$$f_N - x(1 - f_N)^5 = 0 \quad (8)$$

From f_N , the ellipticity for a given C and T can be calculated:

$$\theta_{\text{calc}} = f_N \theta_N + (1 - f_N) \theta_D \quad (9)$$

Hence by fitting θ_{calc} to θ_{obs} measured at different values of C and T , the values of $K_0 = K(T_0)$, and

ΔH can be numerically evaluated. The numerical analyses were performed with Mathematica®.

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