Molecular determinants of tetramerization in the KcsA cytoplasmic domain

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Abstract: The cytoplasmic C-terminal domain (CTD) of KcsA, a bacterial homotetrameric potassium channel, is an amphiphilic domain that forms a helical bundle with four-fold symmetry mediated by hydrophobic and electrostatic interactions. Previously we have established that a CTD-derived 34-residue peptide associates into a tetramer in a pH-dependent manner (Kamnesky et al., JMB 2012;418:237-247). Here we further investigate the molecular determinants of tetramer formation in the CTD by characterizing the kinetics of monomer-tetramer equilibrium for 10 alanine mutants using NMR, sedimentation equilibrium (SE) and molecular dynamics simulation. NMR and SE concur in finding single-residue contributions to tetramer stability to be in the 0.5 to 3.5 kcal/mol range. Hydrophobic interactions between residues lining the tetramer core generally contributed more to formation of tetramer than electrostatic interactions between residues R147, D149 and E152. In particular, alanine replacement of residue R147, a key contributor to inter-subunit salt bridges, resulted in only a minor effect on tetramer dissociation. Mutations outside of the inter-subunit interface also influenced tetramer stability by affecting the tetramerization on-rate, possibly by changing the inherent helical propensity of the peptide. These findings are interpreted in the context of established paradigms of protein-protein interactions and protein folding, and lay the groundwork for further studies of the CTD in full-length KcsA channels.

Keywords: structural biology; protein–protein interactions; potassium channels; NMR; sedimentation equilibrium

Introduction

KcsA is a bacterial potassium channel responsible for selective conduction of potassium ions across the cellular membrane.1 KcsA is assembled from four identical membrane-spanning 17 kDa subunits symmetrically arranged around the ion conduction pore. The structure of the KcsA membrane-embedded domain (residues 23–125) was first determined by X-ray crystallography. Each subunit was shown to consist of two membrane-spanning helices (TM1, TM2), and a shorter “pore” helix supporting a five-residue segment which forms the channel selectivity filter, responsible for allowing only K⁺ ions through the pore.2 This structure, together with related ones3,4 has revealed the molecular basis of channel function and regulation. Due to its relative flexibility, the cytoplasmic domain of KcsA (CTD, residues 121–160) was more difficult to characterize structurally. Initial evidence that this region contains a helical domain5-7 was confirmed in the first structures of full-length KcsA determined using Fab antibody fragments as crystallization chaperones.8,9 These and an accompanying structure of a truncated CTD segment (residues 137–158)5 revealed a helical bundle with four-fold symmetry spanning residues 135–155, with key inter-subunit hydrogen bonds and salt bridges involving channel residues H145, R147, D149, and E152 stabilizing the tetrameric structure.
KcsA behaves as a pH-gated channel, switching from a closed conformation at neutral pH to an open conformation allowing passage of K$^+$ ions at acidic pH. Several structural studies have demonstrated the molecular basis of pH-induced opening of the channel, manifested as a bending of the inner pore-lining TM2 helix at a conserved glycine residue. Channel pH-gating is mediated by an interplay of hydrogen bonds in the periplasmic pore-vestibule domain as well as by protonation of negatively charged residues at the base of the aqueous pore where the four inner TM2 helices converge. In this context it was claimed that the tetrameric CTD helical bundle is insensitive to pH, since structures solved at pH 4 and 7 failed to exhibit significant differences in this region. Indeed, the channel maintains its ability to open in a pH-dependent manner in the absence of CTD. However, experimental evidence is contradicting regarding stabilization of the tetrameric structure by CTD residues, claimed in one case to be limited to proximal residues 120 to 124 and in another to include the entire CTD. Furthermore, the interaction energy of crystallization chaperones in the structural studies could offset and mask pH effects on the helical bundle. On the other hand, the notion that the CTD helical bundle is sensitive to pH is supported by several studies. KcsA solubilized in dodecyl maltoside (DDM) micelles or liposomes exhibited a pH effect on CTD structure, and a peptide representing KcsA residues 112 to 160 was shown by sedimentation equilibrium to oligomerize at neutral, but not acidic, pH values. In another study, exchange of acidic residues with neutral ones, particularly of residues E146 and D149, resulted in desensitization of the channel to pH. Recently, a 34-residue peptide representing residues 128 to 160 was shown by NMR and sedimentation equilibrium to exist in monomer-tetramer equilibrium, with a C$_{50}$ value, defined as the concentration at which half the peptide is tetrameric, of 0.3 mM. This study also showed that tetramerization did not occur at pH <5, and this pH-switch was mediated by protonation of key residue H145. Thus, there is considerable evidence that the CTD contains a third pH-sensitive region of the channel.

Protein-protein interactions are a keystone of biological function, accounting for extensive efforts devoted to understanding these on a molecular level as well as to application of established and novel methodologies towards the study of oligomerization of peptides and proteins. Two methods with proven abilities to address such systems are nuclear magnetic resonance (NMR) and the analytical ultracentrifugation method sedimentation equilibrium (SE). In NMR spectral resonance frequencies, or chemical shifts, are highly sensitive to changes in electronic environment which invariably accompany interactions between proteins. More importantly, spectral line-shapes are affected by motions on a wide range of time-scales, allowing kinetic events to be quantitatively characterized. Significantly, both static and dynamic information on the interaction between proteins is obtained in a residue-specific manner, thus localizing protein-protein interactions to a particular structural region. In sedimentation equilibrium (SE) experiments the tug-of-war between two opposing forces, centrifugal displacement and diffusion, creates a dispersion of the protein along the sample from which its molar mass can be extracted. Since a binding event between proteins, forming a homo- or hetero-oligomer, obviously changes the size of the solute, complex formation can be detected in a straightforward manner. Highly complementary to these experimental methods is the quickly-advancing field of molecular dynamics (MD) simulation. MD simulation of proteins, which describes the time evolution of the molecular system, provides atomic details about the protein structure and dynamics. Protein-protein interactions are intertwined by intra- and intermolecular energies as well as solvation effects and entropy. MD simulation is a unique tool to decompose these interactions and provides insights about the determining factor for the protein protein complex formation. Employed together, these three methods are capable of mapping and quantitatively characterizing protein-protein complexes in solution.

In light of evidence establishing the KcsA CTD as an independent tetramerization domain and its potential impact on channel function, in this study we present a detailed description of the molecular determinants of CTD tetramerization. We have studied a series of CTD single alanine mutants using NMR, SE and MD calculations to determine the relative contributions of CTD residues to the stability of the CTD tetramer. Experimental data shows that single residue mutations contribute overall between 0.5 and 3.5 kcal/mol of binding energy to the tetramer. The most significant contribution was found for residues lining the hydrophobic ‘inner’ face of the helical bundle, F148, L151 and L155. A lower contribution was found for the salt-bridge forming triad R147, D149 and E152; surprisingly, the R147A mutant still formed a stable tetramer despite the central role played by this residue in the crystal tetramer structure. A third effect upon tetramer stability was identified, mediated by residues outside of the interaction surface, possibly by affecting the inherent helical propensity of the peptide. This proposed molecular understanding of tetramer stability lays the foundation for further structural and electrophysiological experiments investigating the properties of the KcsA channel and its cytoplasmic pH-sensitive domain.
Results

Application of NMR and SE for studying tetramer stability in CTD34 peptides

Following the results of a previous study,24 we focused on 34-mer C-terminal fragments of the cytoplasmic KcsA domain to investigate the biophysical behavior of this region. The wild-type sequence, (P)H128SEKAAEEAYTRTRGLHERFDRLERMLDDNR160, has been shown to form a four-helix bundle, consisting of a buried hydrophobic surface, a motif of an inter-subunit hydrogen bond and salt bridges involving residues R147 on one helix and H145, D149, and E152 on an adjacent helix, and an exposed surface rich in charged residues.8 In order to determine the relative contributions of various CTD34 residues to tetramer formation we synthesized ten alanine mutants in the region (residues 145–160) shown to be involved in inter-molecular contacts (Fig. 1). Our aim was to apply NMR and SE methods to measure tetrameric stability for each of these mutants, allowing us to determine the individual energetic contributions of various residues to the formation of the tetrameric assembly, and thus formulate the structural principles governing this process. For NMR characterization of monomer-tetramer equilibrium we used the ratio between peaks for the Y137H proton, treating each split doublet as a single peak for purposes of integration. Mutants undergoing intermediate to fast exchange exhibited broadened resonances or a single peak whose position changed in a concentration dependent manner, and so could be distinguished from mutants for which the tetramer concentration dropped below the detection level. In these cases NMR could not be used to determine the tetramer dissociation constant.

Previously used to characterize the oligomerization of wild-type CTD34, here SE was challenged further to provide accurate K_D values for the significantly less stable tetramers formed by mutant CTD34 peptides. Based on Eq. (4) below it is clear that in SE experiments low concentrations of tetramer will be difficult to identify accurately. On the other hand, our prior knowledge of the molecular size of the examined peptide, allowing us to use the known mass of CTD34 to lower the number of fitted parameters, enables the analysis to focus on the oligomerization behavior, facilitating the interpretation of experimental results. Two potential complicating factors in interpretation of the SE data were evaluated and found to have no adverse effects on our analysis. First, since concentrations are measured

Figure 1. The KcsA C-terminal domain four-helix bundle. Shown are two adjacent helices in the CTD bundle (as depicted in the inset) and key residues referred to in the text. Left, back view of the two helices highlighting residues in the hydrophobic core of the helical bundle. Right, front view highlighting residues on the outer surface of the helices. Residues mutated to alanine in this study are shown in stick mode, with hydrophobic, positively- and negatively-charged residues shown in green, blue and red, respectively.
by $A_{280}$ values, potential differences in extinction coefficients between CTD34$^M$ and CTD34$^T$ must be taken into account. Indeed, the relatively weak chromophore in CTD34 (a single Tyr residue) is known to be susceptible to environment effects. However, the $A_{280}$ reading was found to increase linearly with the concentration of wild-type CTD34, whereas nonlinear behavior would be expected if changes in the extinction coefficient of the two CTD34 forms were material. A second concern was possible differences between monomer and tetramer specific volumes, since these depend on the compactness of protein folds and degree of solvation, raising the possibility that the additional buried surface found in tetrameric CTD34 may result in an increase in specific volume. To eliminate this possibility we compared fitted curves using a single specific volume value with those using two different values. This was accomplished using an in-house MATLAB-based least-squares fitting algorithm applied to a function containing two sedimentation terms with distinct sedimentation factors, $\sigma^M$ and $\sigma^T$. In practice this failed to significantly change the obtained $K_D$ values, supporting our assumption that any change in specific volume had a negligible effect on the sedimentation behavior of the sample. For all experiments the specific volume used was 0.71 cm$^3$/g as predicted by SEDNTERP and the value of $\sigma$ used (for experiments at 45,000 rpm) was $1.3 \times 10^{-2}$ mol g$^{-1}$ cm$^{-2}$.

**NMR determination of tetrameric stability of CTD34 mutants**

The aromatic region of the $^1$H-NMR spectrum of CTD34 mutants provided qualitative, and, in some cases, quantitative insight into the kinetics of their tetramerization. Figure 2 compares this region of the spectrum for six representative mutants to that of wild-type CTD34; additional mutants are presented as Supporting Information (Fig. S1). The central defining feature of the spectra is the broadening effect of monomer-tetramer equilibrium upon the observed signals. Four of the examined mutants, E146A, R150A, D157A, and R159A, exhibited peaks representing protons from both monomeric and tetrameric forms of CTD34, similarly to the wild-type peptide. This suggests that the equilibrium between states is slow on the NMR timescale, e.g. the rate of inter-conversion is slower than the difference between monomer and tetramer resonance frequencies. Since the two Y137H$^+$ peaks are ~80 Hz apart at 16.4 T, the rate of dissociation (off-rate) of these tetramers is on the order of s$^{-1}$. At a given concentration, the fraction of tetrameric CTD34 from highest to lowest behaved as D157A > E146A > WT > R159A > R150A. Despite their similar off-rates, tetramer dissociation constants changed between these peptides by over two orders of magnitude (Table I), implying they affected the rate of tetramer association (on-rate). The results observed for the D157A and R159A mutants are particularly interesting, since these residues were shown to be less ordered and not involved in inter-subunit interactions in two unrelated NMR studies, an idea confirmed by the MD results of this study (vide infra).
In contrast to these spectra, other mutants gave rise to spectra with severely broadened peaks, indicating intermediate exchange on the NMR timescale, or a single peak for both monomeric and tetrameric CTD34, evidencing intermediate-to-fast exchange on the NMR timescale. This broadening precluded an NMR-based quantitative determination of the dissociation constant, but the known chemical shift differences between monomer and tetramer peaks (80 Hz for the Y137H shift differences between monomer and tetramer of the dissociation constant, but the known chemical precluded an NMR-based quantitative determination exchange on the NMR timescale. This broadening tetrameric CTD34, evidencing intermediate-to-fast scale, or a single peak for both monomeric and cating intermediate exchange on the NMR time-rise to spectra with severely broadened peaks, indi-

Table I. Tetrameric Stability of Various CTD34 Alanine Mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Notes</th>
<th>$K_D \times 10^{-11} \text{ M}^3$ (by SE)</th>
<th>$K_D \times 10^{-11} \text{ M}^3$ (by NMR)</th>
<th>$C_{1/2} \text{ (mM)}$</th>
<th>$\Delta G \text{ (kcal/mol)}$</th>
</tr>
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<tr>
<td>D157A</td>
<td>Outside helical region</td>
<td>0.5 ± 0.3</td>
<td>0.37</td>
<td>0.22</td>
<td>−0.8</td>
</tr>
<tr>
<td>E146A</td>
<td>Hydrophilic face</td>
<td>0.8 ± 0.4</td>
<td>1.4</td>
<td>0.25</td>
<td>−0.5</td>
</tr>
<tr>
<td>WT</td>
<td>N/A</td>
<td>2 ± 1$^a$</td>
<td>1.9$^b$</td>
<td>0.34</td>
<td>0.0</td>
</tr>
<tr>
<td>R147A</td>
<td>Central to electrostatic motif</td>
<td>5 ± 2</td>
<td>ND$^c$</td>
<td>0.47</td>
<td>0.5</td>
</tr>
<tr>
<td>H145A</td>
<td>h-Bond in electrostatic motif</td>
<td>21 ± 5$^c$</td>
<td>ND$^c$</td>
<td>0.75</td>
<td>1.4</td>
</tr>
<tr>
<td>R159A</td>
<td>Outside helical region</td>
<td>28 ± 7</td>
<td>78</td>
<td>0.83</td>
<td>1.6</td>
</tr>
<tr>
<td>R150A</td>
<td>Hydrophilic face</td>
<td>130 ± 30</td>
<td>228</td>
<td>1.54</td>
<td>2.7</td>
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<tr>
<td>D149A</td>
<td>Ion-pair in electrostatic motif</td>
<td>180 ± 20</td>
<td>ND</td>
<td>1.74</td>
<td>2.9</td>
</tr>
<tr>
<td>E152A</td>
<td>Ion-pair in electrostatic motif</td>
<td>260 ± 60$^c$</td>
<td>ND</td>
<td>&gt;2.0</td>
<td>&gt;3.1</td>
</tr>
<tr>
<td>F148A</td>
<td>Hydrophobic motif</td>
<td>&gt;400$^e$</td>
<td>ND</td>
<td>&gt;2.2</td>
<td>&gt;3.3</td>
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<tr>
<td>L151A</td>
<td>Hydrophobic motif</td>
<td>&gt;550$^f$</td>
<td>ND</td>
<td>&gt;2.5</td>
<td>&gt;3.5</td>
</tr>
<tr>
<td>L155A</td>
<td>Hydrophobic motif</td>
<td>&gt;800$^f$</td>
<td>ND</td>
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$^a$ Based on the SE measurements.  
$^b$ The $C_{1/2}$ value was defined as the concentration at which the populations of CTD34 present as monomer and tetramer were equal, corresponding to $z = 0.5$ in Eq. (1).  
$^c$ $\Delta G = RT \times \ln(K_{mut}/K_{wt})$.  
$^d$ Determined previously, from Ref. 24.  
$^e$ Not determined by NMR due to fast exchange or below-threshold tetramer concentration.  
$^f$ Assuming E152A forms a tetrameric species.  
$^g$ Value defines a lower limit for $K_D$ with 95% confidence. 

In contrast to NMR, SE is capable of analyzing monomer-tetramer equilibrium even under conditions of intermediate or fast exchange, allowing all mutants to be characterized using this method. Figure 3 demonstrates SE curves obtained for selected CTD34 mutants D157A, R147A, R150A and F148A, all affording good global fits to a monomer-tetramer equilibrium model over three different concentrations, and similar results were obtained for the other mutants (Supporting Information, Fig. S2). The sole exception to this was E152A, for which a monomer-dimer equilibrium model offered a slightly better fit, although the significance of this is unclear. For convenient comparison, the behavior of all mutants was simulated for a given concentration using the calculated dissociation constants and a set initial concentration of 0.3 mM. This comparison ranks the various mutants in terms of their tetramer stability, forming a group of more stable tetramers including D157A, E146A and R147A, two mutants with intermediate stability, H145A and R150A, and five mutants forming weak tetramers, including R150A, D149A, F148A, L151A and L155A (Fig. 3 and Supporting Information Fig. S2). Notably, as a comparison to a monomeric single-species curve shows, the equilibrium can be identified even for those with the lowest tetramer stability. As before, changes in the hydrophobic core of the tetramer were the most detrimental to its stability. Due to the extremely low population of the tetrameric species for these mutants, SE determined only a lower boundary for $K_D$ for the dissociation constant. Characteristically, only the overall dissociation constant can be derived from these experiments, whereas two tetramers with very different kinetics, e.g. the slow-exchanging R150A and the fast-exchanging D149A, cannot be distinguished here. By employing analytical centrifugation methods the dissociation constants of six additional mutants could be characterized.
stability of the ten mutants of this study as well as the wild-type and H145A mutant of CTD34 reported in an earlier study. Overall the dissociation constants for the equilibrium between a CTD34 tetramer and four monomers span three orders of magnitude, or, in free energy terms, 4.5 kcal/mol, and good agreement between the two approaches is observed. Ranking by tetramer stability of mutants for which both methods provided $K_D$ values is almost identical, and for mutants exhibiting intermediate-to-fast exchange, thereby precluding an accurate NMR measure of $K_D$, a qualitative agreement is observed, i.e. higher values correlate with faster exchange. These findings can be summarized by three major conclusions, (i) residues contributing most to tetramer stability are in the inner hydrophobic core of the four-helix bundle, (ii) within the network of inter-subunit electrostatic interactions the negatively charged D149 and E152 are the major contributors to tetramer stability, while mutation at H145 and, surprisingly, R147, exhibit a smaller effect, (iii) whereas these two groups of residues affect tetramer dissociation, other mutants giving rise to slow-exchange spectra, e.g. E146, R150, D157 and R159, indicating that they affect the rate of tetramer association.

Circular dichroism confirms the relative tetrameric stability of CTD34 mutants

Secondary chemical shifts indicate a partially helical conformation for monomeric CTD34, and a strong increase in its helicity upon tetramer formation, which can be attributed to selection of the CTD34 helical conformation by the stabilizing effect of the four buried hydrophobic faces of the helical bundle. Therefore, it is expected that circular dichroism (CD) curves will reflect the changes in tetrameric stability between CTD34 mutants. Results for selected mutants are shown in Figure 4. Comparing the F148A, R159A, E146A, and D157A mutants, the first two are expected to exhibit negligible (<1%) tetrameric population, while the last two are 10% and 16% tetrameric, all at CD measurement conditions (60–70 μM). CD curves of the two monomeric mutants exhibit a weak minimum at 222 nm and a

Figure 3. Sedimentation equilibrium experiments for CTD34 mutants. (A) SE curves for (from upper left, clockwise) D157A, R147A, F148A, R150A CTD mutants. Shown are absorbances obtained at a concentration of 0.4 to 0.5 mM peptide, rotation speed of 45,000 rpm and 298 K. Lines represent best fits to a monomer-tetramer equilibrium model with residuals appearing above each curve. (B) Simulated data for comparison of the different mutants. Curves for wild-type CTD34, monomeric CTD34 and 10 mutants simulated at 0.3 mM peptide concentration and a rotation speed of 40,000 rpm. Inset, enlargement of the distal region of the SE curve for monomeric CTD34 and weakly-tetrameric mutants.

Figure 4. Circular dichroism of CTD mutants. Curves acquired in a 0.1 cm cuvette. Mean residue ellipticity is shown for the D157A (black), E146A (dark grey), R159A (grey) and F148A (light grey) mutants. Samples were in 10 mM phosphate buffer pH 7.3 and 283 K.
strong minimum at 200 nm, indicative of a mostly random coil conformation. CD curves of the more tetrameric mutants exhibit a mixture of random coil and α-helix in the case of E146A, and a more helical conformation in the case of D157A. Analysis of the CD curves using the Dichroweb server\textsuperscript{39} uncovers helical fractions of 13% and 11% for the first two mutants, which represent the basal helicity of the monomeric CTD34 peptides, and a higher helicity, 16% and 21%, respectively, for the two latter mutants. Thus, CD results are in agreement with those obtained by NMR and SE on the relative tetramer stability for the various CTD34 mutants.

**MD simulations of energetic contribution to tetramerization**

Having characterized the per-residue contribution to tetramer formation using experimental methods, we set out to examine tetramerization using a computational approach. A 50 ns MD simulation was performed for the KcsA CTD tetramer (residues 129–158) dissolved in water at 298 K. The root mean square deviation (RMSD) of backbone heavy atoms from the X-ray structure (PDB accession code 3EFD) increased to \( \sim 4 \) \( \text{Å} \) after 2 ns and exhibited normally distributed fluctuations in the subsequent 48 ns MD run, suggesting a stable state was reached. The root mean square fluctuation (RMSF) of the backbone C\( \alpha \) atoms of N-terminal residues 129–134 and C-terminal residues 157–158 indicated elevated levels of motion, in contrast to the rest of the protein for which typical RMSF values of \( \sim 1 \) \( \text{Å} \) suggest a more rigid structure (Fig. S3, Supporting Information). These findings are comparable to the crystallographic B-factors,\textsuperscript{8} and suggest that the region of the peptide involved in inter-subunit contacts spans CTD residues 135 to 156.

Binding free energy differences \( \Delta G \) were obtained by comparing the results of the MM-PBSA calculation for wild-type and mutant CTDs. In general MD results exhibit a positive correlation with the SE-derived values (Fig. 5, left) suggesting that the interactions important for tetramer formation are captured in the MD simulation. Interestingly, MD simulations consider residue R147 as a significant contributor to tetramer stability, consistent with the crystal structure but contradicting the experimental results that did not detect a significant weakening of the tetramer for the R147A mutant. This appears to be the exception rather than the rule, suggesting that the MM-PBSA approach fails to correctly represent the behavior of this particular mutant. We also note that generally, the absolute value of predicted \( \Delta G \)s is about nine times larger than the experimental values. This significant overestimation of \( \Delta G \) is surprising considering that the MM-PBSA method reliably reproduced experimental results in a recent protein-protein interaction study.\textsuperscript{40} We attribute this phenomenon to the effects of the auxiliary antibody proteins on the crystallographic tetramer structure.\textsuperscript{8} Presumably, their presence leads to a more densely packed helical bundle

**Figure 5.** MD-derived energetic contributions to tetramerization in various CTD mutants. Left, correlation plot between the \( \Delta G \) values obtained by SE and using the MM-PBSA approach. The best fitted line (excluding the outlier R147A) is \( y = 8.7x - 0.5 \). All differences between experimental and computational energy values are discussed in the text. Energy decomposition of \( \Delta G \) for the mutations F148A, L151A, L155A (upper right) and D149A, R150A, E152A (lower right) to five terms: a) \( E_{\text{ele}} \), b) \( E_{\text{vdW}} \), c) \( E_{\text{int}} \), d) \( G_{\text{sol_nonpolar}} \), and e) \( G_{\text{sol_polar}} \). For definitions of these energy terms see main text.
which may not correctly represent the solution structure that is the focus of this study. Stated differently, inter-subunit interactions are probably weaker than suggested by the crystal structure, and therefore the crystal structure may represent only a small population of the solution tetrameric state. In any event, the general trend in the MM-PBSA results confirms the relative contribution of CTD side-chains to tetramer stability as observed by NMR, SE and CD methods.

We examined the breakdown of energy terms in $\Delta G$ values of six important mutants including F148A, D149A, R150A, L151A, E152A, and L155A, all causing significant decreases in binding affinity, to gain further insight into their contribution to tetramer formation (Fig. 5, upper/lower right). As expected, mutation of hydrophobic residues, e.g. the F148A, L151A and L155A mutants, resulted in a dominant positive contribution of $E_{vdW}$ to the $\Delta G$ values, consistent with the notion that these mutations cause the loss of van der Waals interactions in the tetramer state. In contrast, mutation of charged residues, e.g. the D149A, R150A, and E152A mutants, resulted in mutually compensatory changes in two terms, the electrostatic interaction $E_{ele}$ and the polar solvation energy $G_{sol,polar}$. Mutation to alanine of D149 and E152, whose sidechains form inter-subunit salt bridges with R147, results in a relatively small decrease in the $E_{ele}$ contribution, since the mutant loses less interaction energy than the wild-type, which is offset by a slightly larger increase in polar solvation term. For the exposed R150A the changes in these two terms are much larger; here it is the increase in energy of the $E_{ele}$ interaction which is not fully compensated by a decrease in polar solvation that causes the weakening of the binding affinity. In general, while mutation of charged residues alters the distribution of energy between the different terms, these changes typically do not lead to a significant change in the overall energy. Mutation of hydrophobic residues, on the other hand, leads to a significant differential effect on the contribution of $E_{vdW}$ upon the tetrameric and monomeric states, leading to a more noticeable change in overall energy. This accounts for the stronger effect of such mutations on tetrameric stability, in agreement with the experimental results.

**Discussion**

The structure of the C-terminal domain of the KcsA channel, solved in complex with stabilizing antibodies, filled the gap left by the original KcsA structures that focused on the ion-conducting pore and the selectivity filter. The biological role of this region of the channel is still not fully understood, in particular its involvement in pH-mediated gating of the channel. Our previous study showed that (i) the isolated peptide forms a helical tetramer even in the absence of the membrane-spanning domain, and (ii) this tetramer dissociates at lower pH due to protonation of a key histidine residue. The close relation to the C-terminal tetramer formed by the full channel makes it likely that similar factors govern oligomerization in the two systems. Here we exploited this to understand the relative contributions of various molecular motifs to KcsA tetramerization in the cytoplasm by alanine scanning mutagenesis of KcsA CTD-derived peptides. While it is obvious that membrane-anchoring of the peptides does significantly increase their tendency to form tetramers, we hypothesize that the relative stability of such tetramers will be preserved between the isolated and membrane-anchored systems, justifying our approach for studying channel behavior in the cytoplasm.

The need to work at high concentrations (~0.5 mM) of the relatively small (~4 kDa) peptides in SE experiments raises issues that must be addressed. We have taken care to eliminate the possible effects of change in chromophore absorbance and buoyant molar mass upon formation of the tetramer. One potential difficulty in accurately estimating the $K_D$ is the thermodynamic non-ideality of SE samples at these concentrations, which would lead to an underestimation of tetramer affinity. Another is the use of short sedimentation columns and a high spinning rate, rather than longer columns in conjunction with multiple rotor speeds, which may reduce the quality of acquired SE data. This being said, SE was employed in a comparative manner to study the behavior of several mutants of comparable size and concentration, suggesting that these factors would have a similar effect on all mutants, and most likely leave the general trend reported here unchanged. In addition, the validity of the presented conclusions is strengthened by the agreement observed between all biophysical methods employed in this study.

SE is highly sensitive to the oligomerization state of the solute and afforded an estimate of the dissociation constant $K_D$ for CTD34 mutants in all exchange regimes. In contrast, NMR provided a quantitative measure of $K_D$ only for slow-exchanging mutants, but compensated for this by offering a kinetic view of the equilibrium, allowing the rate of tetramer-to-monomer conversion (off-rate) to be estimated. $K_D$ values calculated by NMR and SE were in good agreement; differences (typically 2-fold, corresponding to ~0.4 kcal/mol) may be attributed to errors in estimating the concentrations of CTD34, which lacks a strong chromophore, based on its absorbance at 280 nm. In this regard, interference measurements may have resulted in improved results. CD measurements provided verification of tetramer fractions in various mutants, exploiting the fact that tetramerization is accompanied by an
increase in helicity. Finally, the MM-PBSA calculations provided an effective tool for predicting the behavior of CTD34 peptides and complementing the experimental results. Although the general experimental trend of relative tetramer stability was captured by these calculations, absolute values for MM-PBSA ΔΔG values were much higher than the experimental ones. We attribute this discrepancy to the fact that inter-subunit distances in the crystal structure may have been influenced by the presence of the stabilizing antibodies, and therefore it does not fully represent the solution structure relevant to NMR and SE experiments. We conclude that the combination of biophysical approaches used in this study provided complementary abilities for investigating formation and stability of mutant CTD34 tetramers.

The dominant feature of the four-helix bundle at the CTD of KcsA is its amphiphilic nature which dictates structure and mode of oligomerization. Our results underline three factors contributing to tetramer stability, (i) a short range hydrophobic effect mediated by hydrophobic residues lining the inner buried surface of the helices, including residues F148, L151, L155 (henceforth the “hydrophobic motif”) and, presumably, L144 which was not included in this study, (ii) a second short-range effect mediated by a network of a hydrogen bond and two salt-bridges formed between R147 of one subunit and H145, D149, and E152, respectively, of an adjacent one (“electrostatic motif”), and (iii) a third effect influencing the tetramer on-rate (as opposed to the previous two affecting the off-rate) including residues E146, R150, D157 and R159. As expected, residues of the first two groups are located at the tetramer interface, whereas residues of the third group are not directly involved in intermolecular contacts, located either on the exposed polar surface of the helices or outside the helix-forming sequence (residues 135–156) as shown in previous studies and confirmed by MD in the present investigation. Our mutagenesis study shows that of the two short-range effects the “hydrophobic motif” is the stronger contributor to tetramer stability (Table I and Fig. 6). A possible interpretation of these findings is that tetramerization is driven by the desire to shield the hydrophobic side of the helices from the solvent, whereas formation of the electrostatic contacts is only a consequence of tetramerization aiming to suffice the energetic needs of charged residues having lost their solvation energy. The parallel between this description and suggested paradigms of protein folding, in which an initial step of formation of a hydrophobic core is followed by a structural ‘fine-tuning’ to accommodate polar groups now lacking water solvation, is striking.

The mechanism by which residues E146, R150, D157 and R159, positioned outside the tetramerization interface, is less evident. The observed on-rate effects may change either the rate of encounters between monomers (typically controlled by diffusion) or the efficiency of such encounters in leading to tetramer formation. Since CTD34 is neutral at our measurement conditions, and mutating negatively or positively charged residues results in opposite effects, electrostatic repulsion between peptides cannot be a significant factor in monomer-tetramer equilibrium. Confirming this is our finding (data not shown) that tetramer stability is insensitive to sample ionic strength. This supports the latter mechanism, e.g. an increase in encounter efficiency, as the dominant factor in the case of CTD34. Since CTD34 helicity is known to increase upon tetramer formation, a plausible hypothesis is that these residues impact tetramer formation by stabilizing the helical conformation, or increasing the helical propensity, of the monomeric peptide. An intrinsically more helical peptide would have a more populated helical conformation, correctly positioning charged and hydrophobic residues for assembly into a helical bundle, thus increasing the probability that four approaching monomers will indeed form a tetramer. Unfortunately, detection of this inherent helicity was not possible by CD, since we could not accurately deconvolute this effect and the helicity resulting from small amounts of tetramer. This hypothesis nicely accounts for effects of the D157A (R159A) mutations, since the decrease in negative (positive) charge at the C-terminal end of the helix in the former case would stabilize (destabilize) the well-known α-helical conformation.
dipole.\textsuperscript{41,42} The situation may be more complicated in the case of the E146A and R150A mutations. Both would be expected to decrease CTD helicity by removing a potential electrostatic interaction between oppositely charged \((i, i + 4)\) side chains, yet the former slightly increases tetramer stability. Without further information we conclude that due to their location in the vicinity of the binding interface, these mutations improve the probability of a fruitful encounter between monomers by a yet unclear mechanism. Curiously, E146 was previously suggested as a key residue in pH-gating of the channel, and the E146Q mutant was claimed to mimic the low-pH state of the channel.\textsuperscript{43} Since mutation to alanine in our study shows only a small effect on tetramer stability, the data presented here do not support this hypothesis.

Returning to the importance of the ‘electrostatic motif’ for tetramer formation, our study differentiates between negatively charged residues D149 and E152, significant contributors to tetramerization, and residue R147, whose contribution to tetramerization is surprisingly small considering its involvement in three inter-subunit contacts. A possible explanation lies in the position of these three residues on the CTD34 helical wheel representation (Fig. 6). Of these three residues R147 is anomalously located within the hydrophobic face of CTD34, and its positive charge buried upon tetramer assembly is highly unfavorable when its balancing counter-charges, D149 and E152, are absent. In contrast, the R147A mutation removes this charge from the tetrameric core while introducing a helix-promoting residue. Concomitantly, residues D149 and E152, previously recruited into the tetrameric core by the electrostatic requirements of R147, may now assume their more natural solvent-exposed conformation as part of the charged face of the amphiphilic helix without any significant energetic penalty. This implies that the R147A adopts a different conformation than wild-type CTD34, explaining chemical shift changes observed in its aromatic spectrum (Fig. 2). It also accounts for the discrepancy between MD and experimental results for this mutant; while the MM-PBSA approach reflects the loss of the electrostatic interaction between R147 and the negatively charged residues, it does not consider possible rearrangements which allow their solvation and limit this loss. This emphasizes the role of R147 in correctly orienting the channel subunits in the cytoplasmic domain rather than driving their association, a task reserved for the hydrophobic residues lining the amphiphilic helices.

In conclusion, we have systematically probed the contribution of residues along the KcsA cytoplasmic domain to the tetrameric stability of the channel. In line with accepted paradigms of protein folding, hydrophobic residues buried in the helical bundle upon tetramerization are the strongest contributors to tetramer formation. However, a network of inter-subunit electrostatic contacts is important for association, particularly in directing the relative orientation of the four helices. An additional contribution is observed for the inherent helicity of the CTD sequence which leads to more efficient association, emphasizing the correlation between propensities for helical conformation and tetramerization. These biophysical considerations determine the oligomerization behavior of KcsA in the cytoplasm, underlining their importance in future studies of channels from both functional and mechanistic points of view.

Materials and Methods

NMR sample preparation

Peptides investigated in this study were derived from the KcsA C-terminal sequence, including the final 33 residues of the channel subunit (128–160) preceded by a non-native proline residue. Peptides consisting of these 34 residues (CTD34) were synthesized by solid-phase Fmoc-based synthesis (EZBiolabs, CA) and further purified on a C8 HPLC column (Phenomenex, Torrance, CA, USA) using an acetonitrile:H2O gradient. The D149A and E152A mutants were recombinantly expressed and purified as described previously.\textsuperscript{24} Samples were prepared by dissolving lyophilized peptide in (unless stated otherwise) 20 mM phosphate buffer (pH 7.3) and 20 mM NaCl. NMR samples were prepared in 99.9% \(^2\)H2O, and SE samples were prepared in H2O.

Acquisition of NMR data

All NMR measurements were conducted on a DRX700 Bruker spectrometer using a cryogenic triple-resonance TCI probehead equipped with z-axis pulsed field gradients. One dimensional \(^1\)H-spectra for concentration-dependent data used to determine the dissociation constant were acquired for samples with varying CTD34 concentrations of 0.1 to 3.5 mM as needed for each mutant. All experiments were conducted at 16.4 T and 298 K. Chemical shifts for backbone nuclei and protons in the aromatic region of monomeric and tetrameric CTD34 forms were referenced directly against 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). Spectra were processed, phased and corrected for baseline artifacts before integration of peak intensities.

For analysis of NMR data we assumed that for CTD34 in equilibrium between monomeric and tetrameric forms, where \(\alpha\) is the fraction of CTD34 in monomeric form and \(C\) is the overall CTD34 concentration, Eq. (1) must hold:

\[
\frac{K_D}{4C^3} = \frac{x^4}{1 - x}
\]

where \(K_D\) is the dissociation constant for the conversion of a tetrameric assembly into four monomeric peptides. The ratio of integrals between monomer
and tetramer peaks representing single protons was assumed to reflect the z/(1 - z) ratio, since each tetrameric molecule gives rise to four times the signal of the monomer, allowing $a$ to be calculated for each CTD34 concentration. The two $^1$H-doublets originating from the H$^\alpha$ nuclei of residue Y137 resonating at 6.72 (of tetrameric CTD34) and 6.84 ppm (of monomeric CTD34) were used to determine the monomer:tetramer ratio in CTD34. For simplicity, each doublet was treated and integrated as a single peak. The value of $K_D$ was then extrapolated from by fitting the dependence of $a$ upon the concentration to Eq. (1). The $C_{iD}$ value was defined as the concentration at which the populations of CTD34 present as monomer and tetramer were equal, corresponding to $z = 0.5$ in Eq. (1). Concentrations were determined by spectrophotometry using an extinction coefficient of $e = 1,490$ M$^{-1}$ cm$^{-1}$, and care was taken to maintain constant buffer conditions. Acquisition times were adjusted to obtain a similar and satisfactory (>100:1) signal-to-noise ratio in all spectra. The standard deviation of three measurements of peak integrals was used as an estimate for the experimental error.

**Sedimentation equilibrium experiments**

Sedimentation equilibrium experiments on CTD34 peptides in the above buffer were performed using an XL-1 analytical ultracentrifuge equipped with the An-60Ti rotor and absorbance optics (Beckman-Coulter Inc., Brea, CA). Data (absorption at 280 nm) were collected at 298 K in double-sector cells of 12 mm thickness. Sedimentation curves were recorded after spinning at 40,000 to 45,000 rpm for 10, 12, and 14 h, typically affording almost identical results (standard deviations of 0.004 A$^2$80 units, or 0.5–2%), suggesting equilibrium had been reached. A single run was performed for each mutant, and the final data set was used for analysis. This experiment was conducted simultaneously for three different CTD34 concentrations, typically in the 0.6–2.4 mg/mL (0.15–0.6 mM) range, and these results were used to globally fit the sedimentation parameters. The relevant equations for the M-T exchange of a CTD34 peptide are

$$4M \rightarrow T \quad K_D = \frac{[M]^4}{[T]}$$

where $M$ and $T$ represent monomer and tetramer, respectively, and $K_D$ is the dissociation constant. The balance of centrifugal field and diffusion effects at equilibrium on a particular molecular species $i$ is given by

$$C_i(r) = C_0^i \exp\{\sigma M (r^2 - r_0^2)\}$$

in which $C(r)$ is the concentration at radial distance $r$, $C_0^i$ is the concentration at the cell edge which is at radial distance $r_0$, and $M$ is the molar mass. $\sigma$ is the sedimentation factor excluding the molar mass which appears explicitly, and equals $(1 - v_p)\rho a^2/2RT$, where $v$ is the specific volume, $\rho$ is solution density (calculated as 0.999 g/cm$^3$ for measurement conditions), $\omega$ is the radial velocity in rad/s, $R$ is the gas constant, $8.31 \times 10^\gamma$ erg deg$^{-1}$ mol$^{-1}$, and $T$ is the temperature. Considering the sum of concentrations of both monomeric and oligomeric species, and substituting the equilibrium equation, the effect of the centrifugal field on concentration is given by:

$$C(r) = C_0^M \times \exp\{\sigma M (r^2 - r_0^2)\} + (1/K_D)[C_0^D]^{1/4} \times \exp\{\sigma (4M)(r^2 - r_0^2)\}$$

Fitting of the SE curve of concentration (represented by the measurable $A_{280}$) as a function of distance from the axis of rotation will thus yield values for $C^i$, $K_D$, and $M$. The specific volume was calculated based on the amino acid sequence using SEDNTERP (http://sednterp.unh.edu/), and found to be 0.706 to 0.712 cm$^3$/g for the various CTD34 peptides. The average curves measured for three concentrations were fitted globally with the SEDPHAT software package.

**Circular dichroism experiments**

CD experiments were acquired on a Chirascan polarimeter (Applied Photophysics, Surrey, UK) for CTD34 samples in the 30 to 70 $\mu$M concentration range placed in a 0.1 cm cuvette. Typical conditions were 10 mM KPi buffer at pH 7.3 and 283 K. Each experiment was acquired as a triplicate, averaged, and subtracted from a measurement of an identical buffer sample. Results were analyzed using the CDSSTR module of the DichroWeb platform for the 190 to 240 nm range.

**MD simulations of the KcsA CTD**

MD simulations were carried out using Gromacs 4.5,45,46 the amber ff99SB force field47 and TIP3P water. The starting coordinates were from the X-ray structure of the KcsA C-terminal domain (pdb 3EFD)8 after removal of the auxiliary antibodies. All residues were assumed to be in their standard ionization states at pH 7.0. The protein was solvated by adding 10.0A TIP3P water in a rhombic dodecahedral box with counter ions added to neutralize the system. The Particle-Mesh-Ewald method48,49 was used to evaluate the contributions of the long-range electrostatic interactions. A non-bonded pair list cutoff of 10.0 Å was used and updated every five steps. All bonds to hydrogen atoms in the protein were constrained by using the LINCS algorithm50 whereas bonds and angles of water molecules were constrained by the SETTLE algorithm51 allowing a
time step of 0.002 ps. The temperature was controlled by a modified Berendsen thermostat.\textsuperscript{52}

Details of the MD simulation are as follows. First, the system was minimized for 5000 steps with all heavy atoms restrained by the harmonic potential \( k = 10 \text{ kJ/mol} \cdot \text{Å}^2 \). The system was heated gently from 50 to 300 K in a 10 ps NVT simulation, after which a 2 ns NPT (1 atm, 300 K) MD simulation was performed. In both simulations, the backbone heavy atoms were restrained. Then, a fully relaxed NPT MD simulation was performed for 50 ns. The snapshots were saved every 100 ps, with the first 2 ns simulation treated as the equilibration thus not included in the data analysis. For the monomeric state, GXG model was built where X was the residue type that was mutated in the experiment. Similar simulation protocols were employed for the GXG model solvated with TIP3P water. A total of 1.5 ns MD simulation was performed for each GXG and 50 MD snapshots were saved for the last 500 ps.

**MM-PBSA calculation**

MM-PBSA alanine scan calculations\textsuperscript{53,54} were performed using the mm-pbsa module in the amber 11 software suite.\textsuperscript{55} The total free energy of a molecule is written as:

\[
G_{\text{mol}} = E_{\text{int}} + E_{\text{ele}} + E_{\text{vdW}} + G_{\text{sol,polar}} + G_{\text{sol,nonpolar}} - TS
\]  

(5)

where \( E_{\text{int}} \) is the internal energy, including the bond, angle and dihedral terms, \( E_{\text{ele}} \) is the intramolecular electrostatic energy, \( E_{\text{vdW}} \) is the van der Waals energy, \( G_{\text{sol,polar}} \) is the polar solvation energy from solving the Poisson Boltzmann equation, \( G_{\text{sol,nonpolar}} \) is the nonpolar solvation energy, and \( TS \) is the entropic contribution. The binding free energy is

\[
\Delta G_{\text{bind}} = G_{\text{tetramer}} - 4G_{\text{monomer}}
\]  

(6)

The binding free energy difference between the wild-type CTD peptide and a given mutant, \( \Delta G_{\text{bind}} \), is defined as

\[
\Delta \Delta G_{\text{bind}} = \Delta G_{\text{mut}} - \Delta G_{\text{wt}}
\]  

(7)

Two approximations were introduced in the calculations. First, the entropic contribution to the \( \Delta G_{\text{bind}} \) was neglected by assuming that it canceled out between the wild-type and the mutant.\textsuperscript{52} Second, for the KcsA monomeric state which is disordered, a tri-peptide GXG was introduced to mimic the structure where X is the residue type at the mutated site. Dielectric constants of 4 (80) were applied for the protein (water) in the Poisson Boltzmann calculation. The nonpolar contribution to the solvation free energy was modeled as a term dependent on the solvent accessible surface area (SASA) of the molecule.\textsuperscript{56} The side chain of the targeted residue was mutated to an alanine while the rest of the system remained unchanged for both the tetramer and the monomer (GXG). The calculations were carried out for the saved MD snapshots and averaged to yield binding free energy difference using Eq. (7), from which contributions from individual terms in Eq. (5) could also be obtained.

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