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Thermodynamics, kinetics, and salt-dependence of folding of YopM, a large leucine-rich repeat protein

Ellen Kloss and Doug Barrick*

Abstract

Small globular proteins have many contacts between residues that are distant in primary sequence. These contacts create a complex network between sequence-distant segments of secondary structure, which may be expected to promote the cooperative folding of globular proteins. Although repeat proteins, which are made up of tandem modular units, lack sequence-distant contacts, several of considerable length have been shown to undergo cooperative two-state folding. To explore the limits of cooperativity in repeat proteins, we have studied the unfolding of YopM, a leucine-rich repeat (LRR) protein of over 400 residues. Despite its large size and modular architecture (15 repeats), YopM equilibrium unfolding is highly cooperative, and shows a very strong dependence on urea concentration. In contrast, kinetic studies of YopM folding indicate a mechanism that includes one or more transient intermediates. The urea dependence of the folding and unfolding rates suggests a relatively small transition state ensemble. As with the urea dependence, we have found an extreme dependence of the free energy of unfolding on salt concentration. This salt dependence likely results from general screening of a large number of unfavorable columbic interactions in the folded state, rather than from specific cation binding.

Keywords

Repeat-protein; leucine-rich repeat; cooperative folding; electrostatics; folding kinetics

Introduction

The study of repeat protein folding has contributed substantially to our understanding of protein folding thermodynamics, cooperativity, stability distribution, and folding kinetics^{1,2}. Repeat proteins are constructed from individual units of supersecondary structure (e.g. $\alpha\alpha$, $\alpha\beta$, $\beta\beta\beta$ for ankyrin [ANK] and tetratricopeptide [TPR] repeats, leucine-rich repeats, and β -prism proteins, respectively) that pack together regularly in a roughly linear array. This simple linear architecture facilitates comparison of different regions of the polypeptide in terms of contribution to stability, cooperativity, and involvement in rate-limiting steps in folding.

Compared with globular proteins, repeat proteins lack close contacts involving distant regions of the polypeptide. This local and regular topology further contributes to what appears, at the structural level, to be a high degree of modularity. Given this architectural

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*Correspondence: barrick@jhu.edu, (410) 516-0409, T.C. Jenkins Department of Biophysics, Johns Hopkins University, 3400 N. Charles St., Baltimore MD 21218 USA.

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modularity, the experimental observation that many repeat proteins (in particular ANK repeat proteins with as many as six structured repeats) unfold by a cooperative two-state mechanism has been somewhat surprising³⁻⁹. Moreover, a recent analysis of consensus ANK repeats with a nearest-neighbor Ising model confirms a very high level of cooperative unfolding¹⁰, although similar analysis of consensus TPR repeats shows lower cooperativity^{1,2,11}.

In comparison with helical repeat proteins, there are fewer studies on the equilibrium folding and cooperativity of β -sheet-containing repeat proteins despite their widespread taxonomic distribution*. Studies on InlB, which contains seven LRR units, show a cooperative two state equilibrium and kinetic folding mechanism^{3,4,12}. However, consensus-based LRRs show substantially less cooperativity in equilibrium folding¹². In addition, pelC and pertactin, two β -prism repeat proteins, exhibit multistate equilibrium folding^{13,14}.

Given the structural differences between LRR and ANK repeat proteins, especially at the interfaces between repeats, which are likely to contribute to cooperativity, a better characterization of the degree to which LRR proteins can maintain cooperative equilibrium unfolding is essential. As illustrated in studies of a large fragment of ankyrinR (D34) containing 12 ANK repeats (426 residues), one way to probe the limits of cooperativity is to characterize unfolding of large repeat proteins¹⁵. For D34, clear multistate unfolding behavior was observed, in which the N-terminal repeats unfold at low denaturant concentration, leaving an equilibrium intermediate that retains structure over the C-terminal repeats.

To better understand the thermodynamic and kinetic properties of LRR proteins, and to explore the limits of cooperativity at long chain length, we monitored the equilibrium unfolding of YopM, a 15 LRR-containing bacterial virulence factor from *Yersinia pestis*. We evaluated the equilibrium unfolding mechanism for YopM by comparing urea- and thermally-induced unfolding transitions monitored with various spectroscopic methods, and by differential scanning calorimetry. We show that a high degree of cooperativity can extend over a large, elongated (80 Å) leucine-rich repeat protein. We find the free energy of unfolding to show extreme sensitivity to salt concentration. We have examined the origin of this salt dependence by comparing the effects of two different chloride salts, NaCl and NH₄Cl. Our results suggest this salt dependence arises from a large number of columbic interactions in the native state. In contrast to the simple two-state equilibrium unfolding mechanism, the kinetic mechanism of unfolding and refolding is multistate, as has been seen for several smaller repeat proteins¹. The high sensitivity of the equilibrium unfolding reaction to urea is partitioned largely into unfolding kinetics, supporting a small transition state ensemble.

Results

The crystal structure of YopM (Figure 1) shows an extended architecture that resembles a horseshoe with a slight helical twist¹⁶. The individual LRR units, 20 or 22 residues each, form β -strands that contribute to a parallel sheet along the concave surface. The residues opposite to the β -sheet, on the convex surface, are in a combination of coil and extended polypyrrolone II conformations, as is typical of bacterial-type LRRs (Figure 1C)¹⁷. In addition to the β -sheet, YopM contains an N-terminal α -helical capping motif with two tryptophans and two tyrosines. Seven additional tyrosines are distributed over the LRR domain (Figure 1A), and one tyrosine is in the unstructured C-terminus. The two tryptophans are mostly

*LRRs are, for example, the second-most common sequence motif in PFAM version 21, and are common in bacteria, archaea and eukaryotes.

buried, with zero and eight percent accessible surface area (ASA). Although the two tyrosines in the α -helical motif are similarly buried, the tyrosines in the LRR domain are more solvent exposed, with a range of ASA from 32 to 72 percent (Table S-1).

Structural properties of YopM in solution

To confirm that secondary and tertiary structural features described above are retained in solution, and to establish a starting point for denaturation studies, we collected far- and near-UV circular dichroism (CD) spectra of YopM. The far-UV CD spectrum of YopM contains a single minimum at 217 nm (Figure 2A), characteristic of proteins with high β -sheet content. The spectrum shows little evidence of the α -helical structure, which likely reflects the relative sizes of the helical capping motif (39 residues) compared to the β -sheet containing LRR domain (310 residues).

To examine the extent of tertiary structure around the aromatic residues in YopM, a near-UV spectrum was collected. Optical activity in the near-UV region, often resulting in multiple well-defined peaks (minima or maxima), arises from aromatic side chains, especially those clustered tightly together¹⁸. The near-UV spectrum of YopM contains several peaks (Figure 2B). Based on the high packing density of tryptophan and tyrosine side chains at the N-terminus of YopM (Figure 1A) and the decreased signal of the spectrum when the two tryptophans are substituted with phenylalanine (Figure S-1), it is likely that the N-terminal α -helical capping motif contributes substantially to the near-UV spectrum. Thus, the α -helical capping motif is likely to be well-structured in solution, even though signal from the α -helices is not seen in the far-UV spectrum. Overall, the far- and near-UV CD spectra show YopM to be folded in solution, with extensive β -sheet structure, and tight packing in the vicinity of the aromatic side chains.

To obtain a definitive probe of the N-terminal α -helical capping motif, we monitored fluorescence, which focuses primarily on the N-terminal tryptophan side chains. The fluorescence emission spectrum of folded YopM, following excitation at 280 nm, shows a maximum around 325 nm, with a shoulder at 310 nm (Figure 2C). The short wavelength of the maximum is consistent with burial of the two N-terminal tryptophans in a nonpolar environment. The shoulder is likely to result from the 10 tyrosine residues. In 4 M urea, where YopM is unfolded, the fluorescence near the maximum is quenched substantially and is shifted to 360 nm. However, the shoulder at 310 nm is relatively unperturbed. This difference likely reflects the high sensitivity of tryptophan to its environment compared to tyrosine, and permits us to monitor the fluorescence of the tryptophans in the N-terminal α -helical capping motif, using a 320 nm cut-off filter following 280 nm excitation. Collectively these three spectroscopic probes provide a means to monitor various structural features during equilibrium unfolding.

Urea-induced unfolding transitions of YopM monitored by far-UV CD and fluorescence

To investigate the mechanism of equilibrium unfolding of YopM and to quantify the stability of this large, extended molecule, we performed urea-induced unfolding monitored by far-UV CD and tryptophan fluorescence. This allows us to compare whether different aspects of the protein's structure (β -sheet formation, and the environment surrounding the N-terminal tryptophan residues) are lost simultaneously or in distinct stages.

Urea-induced denaturation of YopM showed a single, sharp unfolding transition monitored by both far-UV CD and tryptophan fluorescence (filled and open symbols, respectively; Figure 3A). The unfolding transitions monitored by CD and fluorescence are coincident, indicating that the β -sheet, which extends over the entire LRR domain, is disrupted to the same extent as the N-terminal α -helical capping motif. Transitions from both probes can be

well-fitted by a two-state (all-or-none) model. High cooperativity of unfolding is supported by the large m -value (Table 2). For proteins that unfold by two-state mechanisms, m -values have been shown to correlate with change in accessible surface area (ΔASA)¹⁹, and thus to chain length. The m -value measured for YopM unfolding ($6.5 \pm 0.3 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{M}^{-1}$) is larger than the expected value ($4.5 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{M}^{-1}$)¹⁹ for a protein that unfolds by such a mechanism. However, a more recent method of predicting m -values using transfer free energies for individual residues (<http://best.utmb.edu/mvalue/index.html#Examples>)^{20,21} gives an m -value of $5.9 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{M}^{-1}$, closer to the observed value.

Thermally-induced unfolding transitions of YopM

To further examine the mechanism of equilibrium unfolding of YopM we performed thermal unfolding studies. In addition to comparing different spectroscopic signals, we can follow thermal unfolding calorimetrically, which provides a rigorous test of the two-state model. As with urea-induced unfolding, thermally-induced unfolding shows a single, sharp transition, which is coincident when monitored by far-UV CD and tryptophan fluorescence (Figure 3B). Fitting a two-state model to the unfolding transitions monitored by CD and fluorescence provides very similar fitted values of the transition midpoint, T_m , and the van't Hoff enthalpy of unfolding, $\Delta H_{vH,T_m}$ (Table 1).

To monitor loss of tertiary structure during thermally-induced unfolding, especially in the N-terminal α -helical capping motif, we also monitored YopM by near-UV CD as a function of temperature (Figure 3C). The intensity of the near-UV CD spectrum decreases as temperature increases from 15° to 55°C. A two-state model fits well to the unfolding transition obtained at 280 nm (near the wavelength of maximum near-UV CD signal), and the T_m is in agreement with the β -sheet transition observed at 217 nm (Table 1). Although the average $\Delta H_{vH,T_m}$ value fitted from the near-UV CD transition is around five percent higher than from far-UV CD or fluorescence, the decreased signal-to-noise in the near-UV CD contributes to a high uncertainty in $\Delta H_{vH,T_m}$. Thus, this difference appears to be statistically insignificant.

To test whether other regions of the near-UV CD spectrum, which might result from separate chromophores in different parts of the protein, are coincident with the transition at 280 nm, we used singular value decomposition (SVD) to analyze the temperature dependence of the entire near-UV CD spectrum. SVD allows the number of independent spectral components contributing to the original spectral series to be estimated and to be separated from noise, which can make a significant contribution to the near-UV CD spectra. The first two components of the decomposition (represented by the product of the singular value and corresponding spectral vector λ_i and v_i , respectively) significantly contribute to the signal and correspond to spectra of the native and denatured states (Figure 3D). The third and fourth spectral components are much smaller in magnitude and are largely featureless. With only two significant spectral components, the transition at 280 nm must reflect a global transition, applicable to the entire near-UV CD spectrum, including wavelengths with lower signal-to-noise.

A classic test of two-state unfolding is a comparison of $\Delta H_{vH,T_m}$, which is model-dependent, to a calorimetrically determined heat of unfolding (ΔH_{cal}), which is model-independent²². We determined ΔH_{cal} for YopM unfolding using DSC. The scans show a single, sharp, and well-defined transition (Figure 3B). Analysis of the unfolding transition using a two-state model gives values of T_m and $\Delta H_{vH,T_m}$ that are similar to those obtained spectroscopically (Table 1). Moreover, ΔH_{cal} is quite close to $\Delta H_{vH,T_m}$, with a ratio of 1.08 ± 0.04 . This calorimetric comparison indicates that YopM unfolds with only two significantly populated equilibrium species--the native and denatured states²².

Refolding and unfolding kinetics of YopM

To investigate whether the apparent simplicity in the equilibrium mechanism of YopM folding is maintained during kinetic refolding and unfolding, and to begin to get insight into the rate-limiting steps in structure formation in this large but structurally simple protein, we have measured the time dependence of refolding and unfolding. Refolding and unfolding reactions were initiated by rapid dilution and addition of urea using a stopped flow apparatus, and the extent of folding was monitored by tryptophan fluorescence. Both the refolding and unfolding reactions show multiple kinetic phases. At most urea concentrations, neither refolding nor unfolding progress curves can be adequately described by a single exponential decay (black curves, Figure 4A, B, and see large, non-random residuals, upper panels). However, both refolding and unfolding curves are adequately described by a double-exponential decay (red curves, Figure 4A, B, and see small, random residuals).

The observation that YopM shows multiphasic refolding kinetics is not surprising, given the large number of prolyl residues in this protein. All 36 of the Xaa-prolyl peptide bonds seen in the crystal structure of YopM are in a *trans* configuration. Although each Xaa-prolyl bond should thus be biased towards the native state isomer in the denatured state (by approximately eight to one)²³, the probability that *all* 36 residues should simultaneously adopt the native configuration is very low (approximately $0.889^{36}=0.014$). The observation that the rate constants for the two refolding phases have small urea dependences is consistent with a contribution from prolyl isomerization²⁴.

To test whether prolyl isomerization in the denatured state is influencing refolding kinetics, we performed “double-jump” refolding experiments^{24,25,26}. YopM was first unfolded in 3.16 M urea for various delay times (0.75 to 10 seconds; long enough for the protein to unfold, but short enough to minimize prolyl isomerization to non-native configurations), and then rapidly refolded by dilution to a final urea concentration of 1.03 M. Double-jump refolding curves at moderate delay times (3–4 sec) are significantly accelerated compared to single-jump refolding. As with single-jump refolding, double-jump refolding appears to be bi-exponential. However, the fast refolding phase in double-jump refolding is faster by a factor of ten than the fastest phase in single-jump refolding at the same urea concentration (Figure 4C; Table S-2, compare k_f and k_2). The slow phase observed in double-jump refolding matches the fast phase in single-jump refolding (Figure 4C; Table S-2, compare k_s and k_1).

This apparent increase in the overall progress of the folding reaction in the double-jump format is consistent with the hypothesis that the refolding from an equilibrated denatured state (e.g. Figure 4A) is limited by prolyl isomerization. This hypothesis is further supported by the observation that the amplitude of the fast phase in the double-jump protocol is dominant when the unfolding delay is short, but decays as the unfolded state is allowed to equilibrate (Figure 4D). In contrast with double-jump refolding of some globular proteins that are limited by prolyl isomerization²⁴, the amplitude of the slow phase of YopM refolding does not show a lag in the double-jump format. We attribute the absence of this characteristic lag phase to the comparatively slow refolding rate ($\sim 0.3 \text{ sec}^{-1}$), which is in the same range as that expected for isomerization of any number of the 36 prolyl residues, and matches the rate constant for formation of the slow phase in the double-jump experiment (0.37 sec^{-1} ; Figure 4D). Kinetic simulations²⁷ using experimental rate constants are consistent with this explanation.

Unlike refolding, the heterogeneity seen in YopM unfolding is not expected to result from prolyl isomerization, especially at high urea concentrations. Whereas the amplitudes of the two kinetic phases in refolding are in the same direction (towards higher fluorescence, as expected from the overall increase in fluorescence signal on folding), the amplitudes for the

two unfolding phases at high urea concentrations (>2.5 M) are of opposite sign. The amplitude associated with the fast unfolding rate constants (A_{-2} , Table S-2) is in the *folding* direction. In the fitted bi-exponential, the fast phase with opposing amplitude results in an apparent lag in unfolding. As discussed below, these kinetic features have been seen in the unfolding of ankyrin repeat proteins, and appear to result from partly structured on-pathway unfolding intermediates^{28,29,30}.

As urea concentration is decreased below ~ 2.5 M, the fast unfolding phase (A_{-2} , k_{-2}) diminishes in amplitude such that only a single unfolding phase is seen (corresponding to the major unfolding phase at higher urea concentrations, k_{-1}). At still lower urea concentrations (below 2 M, near C_m), an additional phase is detected with a very small rate constant (k_{-3} , Table S-2). Unlike the second unfolding phase detected at high urea concentrations, the amplitude of this very slow phase (A_{-3} , Table S-2) is in the same direction as the major unfolding phase, that is, to decreased fluorescence. This phase may result from mixing of prolyl isomerization in the denatured state with slow unfolding, as has been described by Kiefhaber and Schmid³¹.

Salt dependence of YopM unfolding transitions

YopM has a high density of acidic ionizable groups distributed over its surface¹⁶ (Figure 1B). This should result in a high negative charge density at pH values above the pI (calculated to be ~ 4.5), which may be expected to destabilize the native state. If so, YopM should be stabilized by increasing salt concentrations. Indeed, unfolding transitions of YopM in increasing concentrations of NaCl are shifted to higher urea concentrations (Figure 5A). The free energy of unfolding, determined from fitting a two-state model to the transitions, increases from 5.0 to 13.4 kcal $\cdot$ M⁻¹ over the range of around 20 mM to 800 mM NaCl (Table 2).

Salts influence protein stability by three distinct mechanisms: cation binding, electrostatic screening, and Hofmeister effects³². The physical basis for stabilization can be resolved by comparing different salts. Direct binding is expected to be ion specific, since the energetics of binding should depend on the precise stereochemical complementarity of the ion to the binding site. In contrast, charge-charge screening is expected to be nonspecific for ions of the same valence, since the energetics of screening simply requires proximity of the ions to the interacting charges. Like direct binding, Hofmeister effects also show ion-specific effects within the same valence, but follow a well-established trend among the ions³³. Moreover, these mechanisms manifest themselves over different ranges of ion concentration: whereas specific binding can occur at quite low cation concentration, Hofmeister effects extend to 1 M ionic strength and above³⁴.

To determine which mechanisms contribute to the observed salt sensitivity of YopM, we compared the effects of Na⁺ and NH₄⁺, which have the same valence charge, but differ in ionic radius (0.97 Å and 1.43 Å, respectively) and hydrogen bonding potential. Both cations dramatically increase the stability of YopM, shifting the unfolding transition to higher denaturant concentrations (Figure 5A, B) and increasing the free energy of unfolding (Table 2). Below 500 mM, changes in NaCl and NH₄Cl concentrations have identical effects on C_m values, consistent with charge-charge screening rather than direct cation binding. For salt concentrations above 500 mM, where Hofmeister effects become pronounced, the C_m values diverge, with NaCl providing greater stability than NH₄Cl (Figure 5C), in agreement with the relative efficacies of these two cations within the Hofmeister series³³. Our results are consistent with electrostatic screening playing a major role in salt stabilization at concentrations below 500 mM NaCl, a range that includes typical intracellular ionic strengths.

As described by Wyman, the sensitivity of a conformational change to salt concentration can be quantified as a thermodynamic linkage number. In this formalism, the thermodynamic linkage is expressed as the variation of the log of the conformational equilibrium constant with the log of the salt molarity³⁵:

$$v_{NaCl} = \left(\frac{\partial \ln K_u}{\partial \ln [NaCl]} \right)_{T,P}$$

For YopM, the natural log of the equilibrium constant for unfolding is linear in $\ln[NaCl]$ below 500 mM (Figure 6A), permitting the linkage coefficient to be evaluated by a simple linear fit:

$$-\ln K_u = -\ln K_{u,1M} - v_{NaCl} \ln [NaCl]$$

For YopM, 4.2 NaCl equivalents are thermodynamically linked to the folding transition (Figure 6A).

As an alternative means to analyze this linkage coefficient, the fraction of denatured YopM as a function of both NaCl and urea was analyzed by global analysis (Figure 6B). Global analysis yields similar value for the linkage coefficient (v_{NaCl} of -4.1), and is likely to be more robust. The quality of the global fit is high (showing small and random residuals), indicating that salt and urea act independently on YopM stability, as has been seen for a number of globular proteins^{36–40}.

Discussion

Because of their extended, open-ended structures, repeat proteins are excellent candidates for probing the origins and limits of unfolding cooperativity. Across different classes of repeat proteins, a key experimental variable that relates to cooperativity is the number of repeats over which two-state unfolding can be maintained. For α -helical repeats, two-state unfolding is seen for p16^{INK4A} (four repeats^{5–7}), myotrophin (four repeats⁸), and the ankyrin domain of the Notch receptor (six folded repeats⁹), but not for p19^{INK4d} (five repeats⁴¹) or D34 (12 repeats¹⁵). For β -sheet-containing repeat proteins, fewer studies have been published. Although two-state unfolding is seen for InlB (seven repeats^{3,4}), the β -prism proteins pelC (seven repeats¹³) and pertactin (16 repeats¹⁴) show evidence for multistate unfolding. Consensus LRR proteins based on mammalian ribonuclease inhibitor (4–14 repeats¹²) show equilibrium unfolding transitions consistent with a high population of intermediates. These findings may result from a lower cooperativity of β -sheet-containing repeat proteins in general, or for pelC and pertactin, may result from the relatively large sizes. Our findings here with YopM, an LRR protein with 15 repeats, challenge both these explanations.

YopM unfolds by an equilibrium two-state mechanism

Despite its large size, YopM unfolds by a highly cooperative equilibrium transition. Both urea- and thermally-induced denaturation data support a two-state mechanism of unfolding. Spectroscopic probes monitoring the N-terminal α -helical capping motif (tryptophan fluorescence) and the β -sheet of the LRR region (far-UV CD) yield coincident unfolding transitions. The near-UV CD spectrum (monitoring tertiary structure around the aromatic residues) undergoes a temperature transition that matches the far-UV and fluorescence monitored transitions, and can be decomposed to just two components by SVD, supporting a concerted loss of secondary and tertiary structure along the molecule. Moreover, the ratio of

the model-dependent two-state enthalpy of unfolding to the model-independent calorimetric enthalpy is close to unity, indicating that partially folded states are not highly populated²².

Another indication that equilibrium unfolding of YopM is highly cooperative is the steepness of the urea-induced transition, and the large associated m -value. This m -value ($5.7 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{M}^{-1}$ from global analysis of the salt-dependent urea transitions) is one of the largest reported for urea denaturation of a monomeric protein^{19,20} and closely matches that estimated from transfer free energies^{20,21}. Another parameter that is correlated with the size of the cooperative unit is ΔC_p ($5.5 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$). Like the m -value, the ΔC_p for YopM unfolding is quite large. However, it is slightly lower than that expected for a protein of this size ($6.8 \text{ kcal} \cdot \text{mol}^{-1}$)¹⁹. This slightly lower value for ΔC_p may reflect a lower ratio of non-polar to polar ΔASA for YopM (1.08) compared to typical globular proteins (1.29).

Structural origin of cooperativity in YopM

Although previous studies have shown long β -sheet repeat proteins to unfold by multistate equilibrium mechanisms^{12,14}, the present study demonstrates that multistate unfolding is not an inherent property of β -sheet repeat architecture in general. For YopM, the size of the cooperative unit is larger than that of the most cooperative α -helical repeat proteins². Cooperativity in α -helical ANK repeat proteins has been shown to result from a combination of strong stabilizing interfaces and intrinsically unstable single repeat units^{10,42}, as well as a uniform stability distribution over medium length scales⁴³.

To begin to evaluate the relative contributions of the interfaces between LRRs and intrinsic folding of individual units, we calculated the amount of ASA buried at interfaces and the difference in ASA of individual units in the folded and unfolded states¹. The average surface area buried at the interface between neighboring LRR units in YopM is about $1470 \text{ \AA}^2$ ($890 \text{ \AA}^2$ non-polar and $580 \text{ \AA}^2$ polar), similar to values for ANK interfaces ($1480 \text{ \AA}^2$; $1010 \text{ \AA}^2$ non-polar and $470 \text{ \AA}^2$ polar¹). However, ΔASA for folding of individual LRR units ($570 \text{ \AA}^2$, mostly non-polar) is significantly smaller than for ANK repeats ($1510 \text{ \AA}^2$, both polar and non-polar). The modest surface area buried on folding of individual LRR units may reflect greater intrinsic instability compared with single ANK repeats, which would enhance cooperativity by destabilizing partly folded states.

Although intrinsically unstable repeat units can contribute to the high cooperativity in YopM, it does not explain the decreased cooperativity of other β -strand repeat proteins. PelC and pertactin have even more extreme ratios of intrinsic versus interfacial surface areas (around $1900 \text{ \AA}^2$ interfacial compared with to $275 \text{ \AA}^2$ intrinsic), but unfold by multistate mechanisms. However, pelC and pertactin have greater variation in repeat length, and have variable loop insertions. In pertactin these insertions are largest in the N-terminal repeats, which may decrease the stability of this region, leading to the observed multistate transition. Local stability variation has been shown to decrease cooperativity in ANK repeat proteins⁴³.

Kinetic complexity in YopM refolding and unfolding

Studies above demonstrate that equilibrium folding of YopM closely approximates the simplest mechanism imaginable: an all-or-none two-state transition. In contrast, the kinetics of the structural transitions of YopM appear to be more complicated. Both the refolding and unfolding reactions show multiple kinetic phases. Both refolding phases appear to be slowed by prolyl isomerization in the denatured state, limiting the extent to which structural insight can be obtained from refolding. However, unfolding kinetics at high urea concentrations ($\sim 2.5 \text{ M}$) show complexity that is unlikely to result from prolyl isomerization, providing insight into non-trivial aspects of kinetics such as involvement of partly folded states and structure of the transition state ensemble.

The appearance of an apparent lag in the fluorescence-monitored unfolding progress curves has been seen in two ankyrin repeat proteins, the Notch ankyrin domain (six folded repeats) and p19^{INK4D} (five repeats)^{28, 30}. In both cases, this lag is the result of conversion of the native state to an on-pathway kinetic intermediate with native-like fluorescence. For YopM, the fluorescence signal reports on the N-terminal helix cap (Figure 1A), where the two tryptophans are located. If an analogous kinetic intermediate is populated during YopM unfolding, the N-terminus would be expected to remain folded, whereas the C-terminus would become disordered. Analysis of C-terminal deletion constructs should help reveal whether such a kinetic intermediate is formed during YopM folding. Recently, an N-terminal α -helical cap has been shown to become structured in the rate-limiting step in folding of InlB, an LRR protein containing seven repeats⁴⁴.

Another feature of the unfolding kinetics of YopM is the high urea sensitivity of the rate constant for the major unfolding phase (k_{-1}). Although $\log k_{-1}$ varies nonlinearly with urea concentration (either a result of kinetic mixing of microscopic rate constants for separate steps in unfolding, or transition state movement^{45,46}, the average urea dependence of this rate constant can account for most of the (steep) urea dependence of the equilibrium constant for YopM folding. This observation is consistent with substantial structural disruption in the rate-limiting step in unfolding, or alternatively, that the transition state ensemble for folding involves a small number of LRRs (and perhaps the helical cap).

Electrostatics interactions significantly destabilize YopM

Previous studies dissecting repeat protein stability emphasize the importance of local interactions within repeats, as well as nearest-neighbor interfacial interactions in determining stability^{1,2,47}. However, the experiments reported here demonstrate that potential long-range interactions, in the form of electrostatic repulsion, can also make a major contribution to the overall stability of repeat proteins. The free energy of unfolding of YopM increases by over 8 kcal•mol⁻¹ upon addition of about 800 mM NaCl (Table 2). The similarity of NaCl and NH₄Cl in increasing YopM stability supports a model in which the stabilizing effects of both salts result largely from electrostatic screening of unfavorable negative charges distributed over the surface of YopM (Figure 1B).

The increase in stability at low salt concentrations is opposite from the destabilizing effect of salt observed for many globular proteins^{32,48}. In general, favorable charge-charge interactions on the surface of proteins stabilize the native state⁴⁹. Increasing salt concentration shields these favorable interactions, decreasing protein stability. However, because YopM has very high net negative charge (−39 at pH 7.6, assuming the pK_as of ionizable groups are unperturbed), favorable charge-charge interactions are likely to be outnumbered by unfavorable interactions, resulting in stabilization of the native protein at low salt concentrations.

One parameter that quantifies the strength of electrostatic interactions in solution is the Debye length, the distance over which charge-charge interactions are significant. The Debye length depends strongly on the ionic strength of the solvent. Including the contribution of the Tris buffer to ionic strength (~12 mM at a total concentration of 25 mM, pH 7.6) the Debye length at 20 mM NaCl is about 20 Å, and decreases to about 5 Å at 400 mM NaCl. At this distance range, unfavorable negative-negative interactions outnumber favorable positive-negative interactions. Thus, in the range of 20–400 mM ionic strength, where our data suggest nonspecific electrostatic screening, interactions between non-nearest neighbor repeats are likely to contribute substantially to the observed salt effect. The large salt sensitivity of YopM unfolding supports the finding that many weak charge imbalances can significantly perturb the free energy of folding⁵⁰.

A handful of other proteins have been reported to be stabilized by NaCl (Figure 6). These include FynSH3 (54 residues)⁵¹, Bs-CspB (67 residues),⁴⁸ Ribonuclease T1 (RNaseT1, 104 residues)⁵², and apoflavodoxin (afd, 169 residues)⁵³. Like YopM, all of these proteins have net negative charges at neutral pH, with similar charge densities of about -0.1 per residue. The linkage coefficient for YopM (4.1 NaCl equivalents) exceeds values for these proteins (Figure 7). For these proteins, the linkage number increases nonlinearly with chain length and charge, partly as a result of low linkage numbers for the shortest proteins. Although the net negative charge densities of -0.1 per residue are consistent with the stabilizing effect of salt, the exact values of these linkage coefficients are likely to depend on the detailed arrangements of charged groups.

Although linkage coefficients are well-defined thermodynamically, their underlying physical origin can be elusive. Linkage coefficients are sensitive to all types of behavior, including specific binding, electrostatic interactions, and Hofmeister effects⁵⁴. In YopM, there are 66 acidic residues distributed along the length of the molecule (Figure 1B). In some regions, the spacing of the acidic residues is similar to that seen between the phosphates in DNA and RNA (see Figure 1B), leading to a comparable linear charge density. This high negative charge density is common in proteins which act as DNA mimics⁵⁵. Increased charge repulsion is thought to destabilize double helix formation in DNA, and is likewise expected to destabilize the folded state of YopM⁵⁴. For DNA the addition of salt stabilizes the duplex form by a polyelectrolyte effect in which cations accumulate nonspecifically around the DNA. This preferential interaction gives rise to a linear relationship between the free energy of duplex formation and the log of the salt concentration (i.e. constant linkage coefficient) over the same broad range of salt concentration seen here for YopM⁵⁴.

Like the large denaturant dependence of YopM, the large fitted linkage coefficient is consistent with a highly cooperative unfolding transition, although the mechanisms by which salt and urea affect protein stability are quite different. Salt stabilizes YopM by charge-charge screening in the native state, whereas urea destabilizes YopM by favorable interactions with the backbone in the denatured state²⁰.

In addition to free energy of unfolding, the m -value has a salt dependence above 500 mM NaCl, although no significant salt sensitivity below this concentration (Table 2). Although a decrease in m -value can result from conversion to a multistate equilibrium mechanism⁵⁶, we find CD- and fluorescence-monitored unfolding transitions to be coincident up to 800 mM NaCl (Figure 5A), suggesting that the two-state character of the YopM unfolding transition is retained. This decrease in m -value is likely to result from a decrease in surface area upon unfolding in higher salt. A more compact denatured state would be favored by screening unfavorable electrostatic interactions at high salt concentrations, which would decrease the ΔASA ^{57,58}. Electrostatic interactions have been reported to affect the denatured state in several proteins^{57,59–67}.

The unique molecular architecture and electrostatic properties of YopM make it an attractive model system for studying long- and short-range electrostatics. The concave surface provides a geometry to study long-range charge–charge interactions through solvent, unlike globular proteins. The similarity between the units composing YopM provides many instances of unfavorable electrostatic interactions that can easily be manipulated experimentally and computationally to better understand charge-charge interactions on protein surfaces.

Materials and Methods

Protein expression and purification

A yopM expression construct, based on the pMal-C2 vector (New England Biolabs) was obtained as a gift from D. Waugh. The purification was performed as described previously¹⁶, with minor changes. After the last purification step, YopM-containing fractions of greater than 95% purity were dialyzed into storage buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.6, 0.1 mM TCEP), passed through an 0.22 µm filter, flash-frozen, and stored at -80°C. Protein concentrations were determined as described by Edelhoch⁶⁸.

Fluorescence spectroscopy

Fluorescence emission spectra were collected on an Aviv ATF 105 spectropolarimeter (Lakewood, New Jersey), in a 1.0 cm cuvette. Protein concentrations were of 4.5 µM, in storage buffer with 0 or 4.1 M urea, at 25°C.

CD spectroscopy

CD spectra were collected on an Aviv 62A DS spectropolarimeter (Lakewood, New Jersey) and a Jasco J-810 spectropolarimeter (Easton, MD). Far- and near-UV CD spectra were collected in 0.1 and 1.0 cm cuvettes with protein concentrations of 18 and 20 µM, respectively, in storage buffer at 25°C. Spectra were obtained by averaging five scans, each with a five second signal average every 1.0 nm.

Two-state analysis of equilibrium unfolding transitions

Equilibrium unfolding transitions induced by urea and temperature were related to the equilibrium constant for unfolding assuming a population-weighted average of the signal of the native (Y_N) and denatured (Y_D) states:

$$Y_{obs} = f_N Y_N + f_D Y_D = \left(\frac{1}{1+K_u} \right) Y_N + \left(\frac{K_u}{1+K_u} \right) Y_D \quad (1)$$

Where, f_N and f_D are the fraction of native and denatured protein, and K_u represents the equilibrium constant for unfolding. Y_N and Y_D are assumed to have linear dependence on urea and temperature and are represented by a lines: $Y_N = a_N + b_N \cdot x$, and $Y_D = a_D + b_D \cdot x$. The equilibrium constant for unfolding is related to the reaction free energy by the standard formula:

$$K_u = \exp \left(\frac{\Delta G_u^\circ}{RT} \right) \quad (2)$$

where, R is the universal gas constant and T is absolute temperature. The two-state model was fit to data from CD and fluorescence signal was expressed by combining equations (1) and (2):

$$Y_{obs} = \frac{(a_N + b_N x) + (a_D + b_D x) \exp(-(\Delta G_u^\circ)/RT)}{1 + \exp(-(\Delta G_u^\circ)/RT)} \quad (3)$$

For urea- and thermally-induced unfolding, the appropriate expressions for ΔG_u° (see below) were substituted into equation (3), which was fitted to the transitions using the nonlinear least-squares tool of Kaleidagraph 3.4 (Synergy Software).

Urea-induced unfolding

Urea-induced unfolding was monitored by CD at 217 nm and total tryptophan fluorescence on an Aviv 62A DS titrating CD spectrometer. Urea purchased from Amresco (Solon, OH) was stirred with mixed-bed resin (Bio-Rad; Hercules, CA) as described in Street et al.⁶⁹, and concentration was determined by refractometry⁷⁰. Urea titrations were carried out automatically using a computer controlled Microlab syringe titrator (Hamilton Company, Reno, NV) to deliver buffered urea solutions (containing protein) into a buffered protein sample. At each urea concentration, samples were equilibrated for 5 minutes at 25°C, and CD and fluorescence signals were averaged for 30 seconds. Protein concentrations ranged from 0.5–3.2 μM. Buffers containing NH₄Cl were adjusted to a measured pH of 7.6.

Thermodynamic parameters were estimated using the linear extrapolation method^{36,70}, in which the free energy of unfolding varies linearly with urea concentration to the data though the equation^{70,36,71}:

$$\Delta G_u^\circ = \Delta G_{u,H_2O}^\circ - m[\text{urea}] \quad (4)$$

Equation (4) was inserted into equation (3), which was then fitted to the data.

Thermally-induced unfolding monitored by far-UV CD

Thermally-induced unfolding was monitored by far-UV CD at 217 nm from 15°C to 60°C, in 1°C steps. At each temperature the sample was equilibrated for 1.5 minutes. Samples were prepared by buffer exchange to thermal unfolding buffer (20 mM NaCl, 20 mM Tris-HCl pH 7.6, 0.05 mM TCEP). Protein concentrations were between 0.5 and 1.5 μM.

Thermodynamic parameters for thermal unfolding were obtained by fitting equation (3) to the thermally-induced unfolding transitions, assuming the following temperature dependence of the free energy of unfolding:

$$\Delta G_u^\circ = \Delta H_{vH,T_m} \left(1 - \frac{T}{T_m} \right) + \Delta C_p (T - T_m) - T \Delta C_p \ln \left(\frac{T}{T_m} \right) \quad (5)$$

Values of $\Delta H_{vH,T_m}$, and T_m were optimized during the fit, whereas the value of ΔC_p was fixed to that obtained from DSC.

Thermally-induced unfolding monitored by near-UV CD

Near-UV CD spectra were collected from 350 nm to 240 nm at a continuous scan rate of 20 nm per minute, with an averaging time of 8 seconds. For full spectral analysis of thermal unfolding, spectra were collected every 2°C, whereas for single-wavelength analysis (280 nm, the maximum in the near-UV spectrum), ellipticity was measured in increments of 1°C. Samples were thermally equilibrated for 1.5 minutes at each temperature. Samples contained 15 μM YopM in thermal unfolding buffer. CD signals were normalized by concentration and path length, but not by number of residues. Spectra were baseline-corrected and were offset to zero at 350 nm. To avoid contribution from the far-UV signal, scans were not analyzed at wavelengths shorter than 265 nm. Singular value decomposition (SVD) was carried out using Matlab (Mathworks; Natick, MA).

Differential scanning calorimetry

DSC was performed from 15°C to 60°C, at a scan rate of 45°C per hour with a Microcal VP-DSC (Northampton, MA). Protein samples were exchanged extensively into thermal

unfolding buffer using a VivaSpin 500 microconcentrator (Sartorius; Aubagne, France) and were scanned against thermal unfolding buffer at protein concentrations ranging from 7.5 to 15 μ M.

To obtain the calorimetric enthalpy, ΔH_{cal} , numerical integration was performed on DSC transitions that had been baseline corrected using by the progress baseline tool of the Microcal Origin Software. To obtain a model dependent van't Hoff enthalpy from the DSC data, the excess heat capacity, (δC_p), was represented by the following equation:

$$\delta C_p(T) = C_{pN} + \left(\frac{K_u(T) \Delta C_p}{(1 + K_u(T))} \right) + \left(\frac{K_u \Delta H_{vH}(T)^2}{RT^2 (1 + K_u)^2} \right) \quad (5)$$

where, C_{pN} is the heat capacity of the native protein and is treated as a linear function of temperature. ΔC_p is assumed to be independent of temperature. The temperature dependence of the van't Hoff enthalpy is given by:

$$\Delta H_{vH}(T) = \Delta H_{vH,T_m} + \Delta C_p (T - T_m)$$

Equation (5) was fit to unfolding transitions using proFit (QuantumSoft, Switzerland).

Kinetic unfolding and refolding studies

Fluorescence was monitored on an Applied Photophysics SX.18MV-R stopped-flow rapid mixing device (Leatherhead, UK) using a 305 nm cutoff filter, following a 280 nm excitation (2.3 nm bandwidth). Final protein concentrations were between 2–6 μ M, in storage buffer with 200 mM NaCl. For single jump refolding and unfolding, initial urea concentrations were 3.17 M and 0 M, respectively. Double-jump refolding was started from 0 M urea, increased to 3.16 M urea for various delay times, and subsequently decreased to 1.03 M urea to monitor refolding. Unfolding and refolding amplitudes and rate constants were determined using non-linear least-squares to fit the equation:

$$Y_{obs} = Y_{\infty} + \sum_i \Delta Y_i \exp^{-k_i t}$$

to individual progress curves. Y_{∞} is the fluorescence signal at long times, ΔY_i is the spectroscopic change contributed by the i^{th} phase, and k_i is the rate constant for the i^{th} phase. As described above, two phases were generally necessary (and in all cases sufficient) to reproduce YopM refolding and unfolding kinetics.

Global analysis of urea-induced unfolding in NaCl

Global analysis was performed to simultaneously fit data with NaCl and urea dependence. The free energy in equation (3) was represented as:

$$\Delta G_u^\circ = \Delta G_{H_2O}^\circ - m[\text{urea}] - v_{NaCl} RT \ln([NaCl])$$

and was used to fit all of the unfolding transitions in the data set simultaneously.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The structure of YopM

(A) Ribbon diagram, showing of 15 LRR motifs, which make up the extended β -sheet, and an N-terminal α -helical capping motif (1JL5.pdb¹⁶). Tryptophan (orange) and tyrosine (pink) residues are shown in CPK representation. (B) Surface representation, indicating a high density of acidic residues (red) relative to basic residues (blue). The two views are related by a 180° rotation about the long axis. (C) Typical YopM LRRs (repeats 8 and 9) contain a β -strand (yellow), connected to coil and extended polypyrrolone II region (green) by turns of the β I (gray) and β VII type (purple)⁷². The longer of the two repeats (repeat 8, left) contains a turn of 3_{10} -helix (red).

Figure 2. Solution spectroscopy of YopM

(A) Far-UV CD shows characteristic β -strand signal with a minimum 217 nm. (B) Near-UV CD shows a complex pattern of peaks, consistent with a rigid tertiary structure around at least some aromatic side chains. Near-UV ellipticities are reported per mole of protein and are not normalized to chain-length. (C) Fluorescence emission spectra in buffer (solid line) and 4 M urea (dashed line).

Figure 3. Equilibrium unfolding of YopM

(A) Normalized urea-induced unfolding transitions monitored by far-UV CD (filled symbols, solid lines) and tryptophan fluorescence (open symbols, dashed lines). (B) Thermal unfolding transitions monitored by CD at 217 nm (open squares), CD at 280 nm (open triangles), fluorescence (open circles), and by DSC (diamonds). Initial and subsequent DSC scans (filled and open diamonds, respectively) coincide very closely, indicating remarkable reversibility. Lines in (A) and (B) result from fitting a two-state unfolding model to the data. (C) Thermal unfolding of YopM monitored by near-UV CD spectroscopy at increasing temperatures from 15°C (pink) to 55°C (black). (D) The first four spectral components (λ_1v_1 ; red, green, blue, and black in decreasing contribution to the data) obtained from SVD analysis of the near-UV CD thermal unfolding series (panel C).

Figure 4. Kinetics of unfolding and refolding of YopM

Fluorescence-detected (A) refolding and (B) unfolding of YopM (symbols). Black and red lines show single- and double-exponential fits, respectively; upper panels show residuals. (C) Fitted rate constants as a function of urea. Filled circles, major unfolding phase (k_{-1}); open squares, minor unfolding phase at high urea concentrations (k_{-2}); filled squares, minor unfolding phase at low urea concentrations (k_{-3}); filled upside down triangles, slow refolding phase (k_1 , single jump); filled triangles, fast refolding phase (k_2 , single jump); open triangle, slow refolding phase from double-jump refolding (k_s ; average from 1–7.5 second delay times); filled diamond, fast phase from double-jump refolding (k_f ; average from 1–7.5 second delay times). Solid lines are the results of linear (k_1 , k_2) and quadratic (k_{-1}) fits. (D) Absolute amplitudes of the two phases detected by interrupted unfolding double-jump assay. Open circles, fast phase amplitude (A_f , Table S-2); filled circles, slow phase amplitude (A_s , Table S-2), which matches the rate constant for the fastest phase seen in single-jump refolding (k_2 , Table S-2). Final protein concentrations were between 2–6 μM , in storage buffer with 200 mM NaCl. For single jump refolding and unfolding, initial urea concentrations were 3.17 M and 0 M, respectively. Double-jump refolding was started from 0 M urea, increased to 3.16 M urea for various delay times, and subsequently decreased to 1.03 M urea to monitor refolding.

Figure 5. The salt dependence of the stability of YopM

Representative urea-induced unfolding transitions of YopM monitored by CD (filled symbols) and tryptophan fluorescence (open symbols) in (A) NaCl concentrations of 20 mM (circles), 78 mM (diamonds), 207 mM (squares), 577 mM (upside down triangles), and 805 mM (triangles), and (B) NH₄Cl concentrations of 20 mM (circles), 200 mM (squares), and 803 mM (triangles). Lines are the result of fitting a two-state unfolding model (as described in Materials and Methods) to individual transitions at each salt concentration. (C) The relationship between YopM urea-induced unfolding transition midpoints (C_m , units of molarity), and salt concentration (NaCl, circles; NH₄Cl, triangles). The similar increases in C_m at salt concentrations less than 500 mM support cation-independent screening of unfavorable electrostatic interactions. Deviation of C_m values at higher salt concentrations suggests stabilization by additional cation-specific Hofmeister effects. Error bars are standard errors on the mean.

Figure 6. Wyman linkage analysis of the unfolding reaction of YopM to NaCl molarity
 (A) Linear relationship between the equilibrium constant from urea-induced unfolding and NaCl concentrations, below 0.5 M (correlation coefficient of 0.99). The line is the result of a linear fit, yielding a slope of $v_{\text{NaCl}} = -4.2$. (B) Global analysis of fractional denaturation (monitored by CD, filled circles) as a function of urea and NaCl (below 0.5 M). The fitted surface gives the number of thermodynamically linked equivalents of NaCl as -4.1 , an m -value of $5.7 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{M}^{-1}$ and an extrapolated Gibbs free energy of unfolding as $13.1 \text{ kcal} \cdot \text{mol}^{-1}$ in 1 M NaCl, 0 M urea.

Figure 7. Wyman linkage coefficients for proteins reported to have large NaCl dependent stabilities, as a function of predicted net charge

Net charges were calculated assuming unperturbed pKa values at pH values where stabilities were reported, FynSH3 (FynSH3: 54 residues, -7 net charge at pH 8.1⁵¹; Bs-CspB: 67 residues, -5 net charge, pH 7.0⁴⁸; RNaseT1: 104 residues, -9 net charge, pH 7.0⁵²; afd: 169 residues, -19 net charge, pH 7.0⁵³).

Table 1

Thermal unfolding parameters of YopM monitored by far- and near-UV CD, tryptophan fluorescence, and DSC.

	$\Delta H_{H,Tm}$ (kcal•mol ⁻¹)	T_m (K)	ΔC_p (kcal•mol ⁻¹ •K ⁻¹)	ΔH_{cal} (kcal•mol ⁻¹)	n
Far-UV CD	139.3 ± 3.0	316.8 ± 0.1	n.d. ^a	n.d. ^a	7
Fluorescence	140.0 ± 1.4	317.1 ± 0.1	n.d. ^a	n.d. ^a	3
Near-UV CD	146.4 ± 3.4	316.6 ± 0.2	n.d. ^a	n.d. ^a	3
DSC	138.3 ± 1.8	317.6 ± 0.1	5.5 ± 0.2	128.8 ± 5.1	3

Unfolding enthalpies are evaluated at T_m . To obtain a reasonable estimate of $\Delta H_{H,Tm}$, the value of ΔC_p was fixed to that obtained from DSC. Uncertainties represent standard error on the mean of n independent experiments.

^aNot determined.

Table 2

Thermodynamic parameters of YopM unfolding at varying salt concentrations.

[NaCl] (mM)	$\Delta G^{\bullet}_{H2O}$ (kcal•mol ⁻¹)	m (kcal•mol ⁻¹ •M ⁻¹)	C_m (M)	n
20.4	5.0 ± 0.1	6.5 ± 0.1	0.77 ± 0.01	3
54.2	7.3 ± 0.1	6.8 ± 0.1	1.07 ± 0.03	3
78.18	7.4 ± 0.4	6.5 ± 0.3	1.13 ± 0.02	3
104.4	8.8 ± 0.2	7.0 ± 0.2	1.26 ± 0.02	3
154	10.0 ± 0.2	6.8 ± 0.1	1.48 ± 0.01	3
207	10.7 ± 0.2	6.6 ± 0.1	1.62 ± 0.00	3
292.5	11.2 ± 0.3	6.2 ± 0.2	1.82 ± 0.02	3
412.2	12.4 ± 0.1	6.2 ± 0.3	1.99 ± 0.07	3
577.5	13.1 ± 0.6	5.8 ± 0.1	2.26 ± 0.05	5
805.5	13.4 ± 0.6	5.2 ± 0.2	2.60 ± 0.03	5
[NH ₄ Cl] (mM)				
20	3.9 ± 0.2	5.2 ± 0.2	0.75 ± 0.05	3
38	5.1 ± 0.9	5.5 ± 1.0	0.93 ± 0.04	3
64.1	3.7 ± 0.03	3.8 ± 0.04	0.97 ± 0.02	3
130.7	6.1 ± 1.1	4.7 ± 0.8	1.29 ± 0.02	3
200	7.3 ± 0.8	4.8 ± 0.4	1.52 ± 0.04	3
400.3	8.6 ± 1.2	4.7 ± 0.6	1.84 ± 0.01	4
605	6.3 ± 0.2	3.3 ± 0.2	1.88 ± 0.06	3
803	6.6 ± 0.2	3.2 ± 0.1	2.05 ± 0.06	3

Uncertainties represent the standard error on the mean of n independent experiments.