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Deletion of internal structured repeats increases the stability of a leucine-rich repeat protein, YopM

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ABSTRACT

Mapping the stability distributions of proteins in their native folded states provides a critical link between structure, thermodynamics, and function. Linear repeat proteins have proven more amenable to this kind of mapping than globular proteins. C-terminal deletion studies of YopM, a large, linear leucine-rich repeat (LRR) protein, show that stability is distributed quite heterogeneously, yet a high level of cooperativity is maintained [1]. Key components of this distribution are three interfaces that strongly stabilize adjacent sequences, thereby maintaining structural integrity and promoting cooperativity.

To better understand the distribution of interaction energy around these critical interfaces, we studied internal (rather than terminal) deletions of three LRRs in this region, including one of these stabilizing interfaces. Contrary to our expectation that deletion of structured repeats should be destabilizing, we find that internal deletion of folded repeats can actually *stabilize* the native state, suggesting that these repeats are *destabilizing*, although paradoxically, they are folded in the native state. We identified two residues within this destabilizing segment that deviate from the consensus sequence at a position that normally forms a stacked leucine ladder in the hydrophobic core. Replacement of these nonconsensus residues with leucine is stabilizing. This stability enhancement can be reproduced in the context of nonnative interfaces, but it requires an extended hydrophobic core. Our results demonstrate that different LRRs vary widely in their contribution to stability, and that this variation is context-dependent. These two factors are likely to determine the types of rearrangements that lead to folded, functional proteins, and in turn, are likely to restrict the pathways available for the evolution of linear repeat proteins.

1. Introduction

Determining the contribution of discrete structural units to the overall stability of globular proteins poses a significant challenge. Elongated repeat proteins have simpler linear architectures than globular proteins, allowing the stability contribution of individual supersecondary structural units to be determined through removal and addition of whole repeats [2–4] (Fig. 1). If the removal of repeating units from either end ("terminal" deletion, Fig. 1A) does not disrupt the structure of adjacent repeats, the stability change from deletion provides an estimate of the thermodynamic stability of the deleted repeat, including the single interface it forms with the neighboring repeat [2,3,5]. If the removal of repeating units from the interior ("internal" deletion, Fig. 1B) results in formation of a new interface, the stability change from deletion provides an estimate of the thermodynamic stability of the deleted repeat and the two interfaces it forms with the N- and C-terminal neighboring repeats, offset by the contribution from the new interface. In the Notch ankyrin repeat domain [3], terminal deletions are modestly destabilizing, whereas internal deletions are strongly destabilizing [6].

In *Yersinia pestis* outer protein M (YopM), a repeat protein containing 15.5 leucine-rich repeats (LRRs) from the bacterial subfamily (Fig. 2A), the removal of the C-terminal β-strand cap results in the partial unfolding of adjacent repeats [1]. Further C-terminal deletions demonstrate that internal LRRs 11 and 6 are necessary for structural integrity in adjacent LRRs [1]. YopM sequences from different species and strains of *Yersinia* contain duplications and deletions of LRRs. As a result, these YopM sequences vary in length, from 13 to 21 LRRs [7]. This variability in LRR number indicates that some interfaces are interchangeable in LRR proteins. This finding is consistent with the prevalence of expansion of LRR proteins through internal duplication [8], and with the conservation of terminal regions in variable lymphocyte receptors, which undergo somatic recombination to produce LRR arrays of variable length and composition [9]. However, given the tight packing interactions seen crystallographically between the LRRs of YopM from *Y. pestis* strain KIM (Fig. 2B) [10], such duplications and/or deletions are likely to be selected for stabilizing (or against grossly destabilizing) interfaces.

Here we have created internal deletion constructs of YopM from *Y. pestis* strain KIM to study the relative stability of repeats and their

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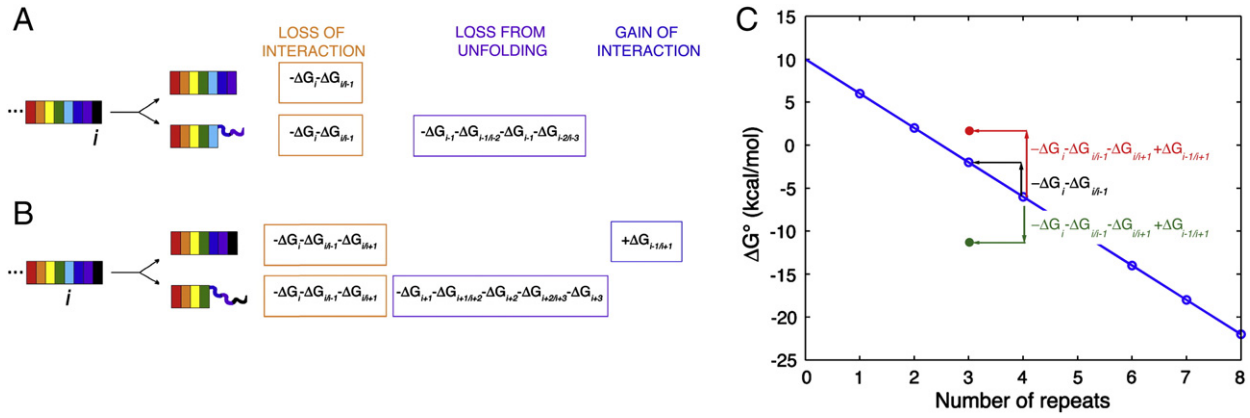
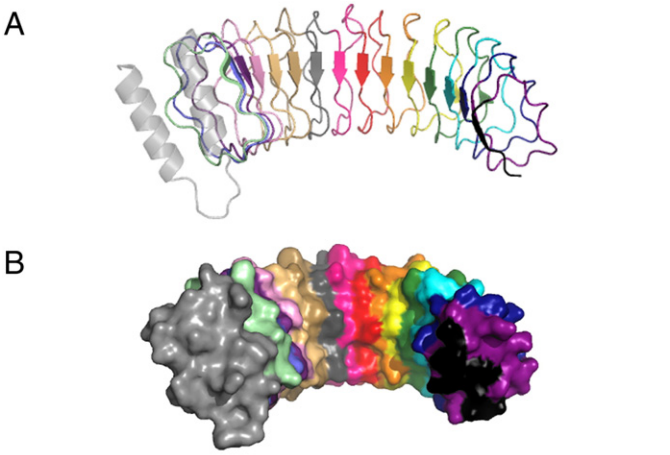


Fig. 1. Thermodynamics of deleting terminal versus internal repeats. (A, B) Schematic diagram comparing (A) terminal versus (B) internal deletion of whole repeats. Changes in folding free energy are on the right, with intrinsic and interfacial terms designated as ΔG_i and $\Delta G_{i/i+1}$, respectively. Energy terms resulting directly from loss of interactions in the fully folded state are listed in orange; terms resulting from partial unfolding (lower lines, A and B) are listed in purple; and the term resulting from forming new non-adjacent interaction ($\Delta G_{i-1/i+1}$) is listed in blue (internal deletion, no partial unfolding). (C) Folding free energy is depicted as a function of repeat number. For the purpose of illustration, each repeat is assumed to have the same intrinsic and nearest-neighbor free energy (blue line). For each terminally deleted repeat, the free energy change is equal to the lost intrinsic ($-\Delta G_i$) and native interfacial energies ($-\Delta G_{i/i+1}$), as indicated with the black arrow. For internal deletion, if the new non-native interface ($\Delta G_{i-1/i+1}$) is less stable than the interface that is lost, the resulting construct is less stable than that created by terminal deletion (red arrow). To get a stability *enhancement* from internal deletion (green arrow), the stability of the new interface ($\Delta G_{i-1/i+1}$) must exceed the sum of the stabilities of all three of the interactions lost (ΔG_i, $\Delta G°_{i/i-1}$, and $\Delta G°_{i/i+1}$).



C

Segment name	Sequence	Segment terminal residue number
LRR 1	HELELNNLGLSSLPEL--PPHL	93
LRR 2	ESLVASCNSLTELPEL--PQSL	113
LRR 3	KSLLVDNNNLKALSDL--PPLL	133
LRR 4	EYLGVSNNQLEKLPELQNSSFL	155
LRR 5	KIIDVDNNSLKKLPDL--PPSL	175
LRR 6	EFIAAGNNQLEELPELQNLPFL	197
LRR 7	TAIYADNNSLKKLPDL--PLSL	217
LRR 8	ESIVAGNNILEELPELQNLPFL	239
LRR 9	TTIYADNNLLKTLPDL--PPSL	259
LRR 10	EALNVRDNYLTDLPEL--PQSL	279
LRR 11	TFLDVSENIFSGLSEL--PPNL	299
LRR 12	YYLNASSNEIRSLCDL--PPSL	319
LRR 13	EELNVSNNKLIELPAL--PPRL	339
LRR 14	ERLIASFNHLAEVPEL--PQNL	359
LRR 15	KQLHVEYNPLREFPDI--PESV	379
β-cap	EDLRMN	385

Fig. 2. Three-dimensional surface and sequence of LRRs in YopM. (A) Ribbon representation of YopM. (B) Surface representation of N- and C-terminal caps and LRRs of YopM (PDB ID: 1JL5 [10]). From left to right the different colors represent different structural units: α-helical cap (gray), LRR 1 (light green), LRR 2 (light blue), LRR 3 (light purple), LRR 4 (light pink), LRR 5 (tan), LRR 6 (brown), LRR 7 (gray), LRR 8 (pink), LRR 9 (red), LRR 10 (orange), LRR 11 (yellow), LRR 12 (green), LRR 13 (teal), LRR 14 (blue), LRR 15 (purple), and β-strand cap (black). (C) Sequence of LRRs in YopM; residues are shaded from light to dark with increasing identity. The solvent accessible surface area in (B) was generated using default settings in Pymol [31].

adjoining interfaces, and to delineate rules that restrict deletion and duplication events leading to natural diversity in LRR arrays. We evaluated the ability of new interfaces to retain structure by circular dichroism (CD) spectroscopy and limited proteolysis, and obtained thermodynamic information from urea-induced unfolding transitions. We find the effects of different internal deletions to be quite variable. Surprisingly, the internal deletion of repeats 11 and 12 actually *increases* stability. To probe the sequence determinants underlying this stability enhancement, substitutions of two non-consensus residues were made in these two repeats (Fig. 2C). These consensus point-substitutions show stability enhancements similar to internal deletion, suggesting that the interaction of these non-consensus residues with their neighboring repeats contributes to the unexpected stability enhancement of internal repeat deletion. Moreover, stability changes resulting from these substitutions underscore the importance of the stacked hydrophobic core for stability, interface compatibility, and for the cooperative folding of YopM.

The broader goals of this research are to contribute to understanding the extent to which biomolecular structure can be resolved into thermodynamic components, to discover the fundamental rules that connect macromolecular structure to thermodynamics (both through generalities and exceptions), and to use these findings to gain insight into biological function, adaptation, and evolution. Answering these broader questions has been a major intellectual driving force for the Gibbs Conferences in Biothermodynamics over the last 25 years. Based on the substantial progress made over the last quarter century by the research groups the Gibbs conference comprises, we expect that answers to these questions will soon be in hand.

2. Results

We have previously mapped the stability contributions of LRRs of YopM by deleting repeats one at a time from the C-terminus [1]. This study showed the stability contributions of individual LRRs to be highly heterogeneous. For example, C-terminal deletion of LRR 13 (as judged by comparing a construct from the N-cap to repeat 13 [N-13] with a construct spanning from the N-cap to repeat 12 [N-12]) has no effect on stability. In contrast, C-terminal deletion of LRR 12 (comparing N-12 with N-11) modestly decreases stability, and C-terminal deletion of LRR 11 (comparing N-11 with N-10) dramatically decreases stability (and results in partial unfolding of adjacent repeats [1]). Here we create internal deletion constructs to probe the relative