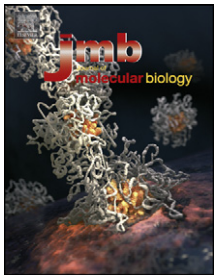

Evolution of Protein Quaternary Structure in Response to Selective Pressure for Increased Thermostability

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Abstract

Oligomerization has been suggested to be an important mechanism for increasing or maintaining the thermostability of proteins. Although it is evident that protein–protein contacts can result in substantial stabilization in many extant proteins, evidence for evolutionary selection for oligomerization is largely indirect and little is understood of the early steps in the evolution of oligomers. A laboratory-directed evolution experiment that selected for increased thermostability in the αE7 carboxylesterase from the Australian sheep blowfly, *Lucilia cuprina*, resulted in a thermostable variant, LcαE7-4a, that displayed increased levels of dimeric and tetrameric quaternary structure. A trade-off between activity and thermostability was made during the evolution of thermostability, with the higher-order oligomeric species displaying the greatest thermostability and lowest catalytic activity. Analysis of monomeric and dimeric LcαE7-4a crystal structures revealed that only one of the oligomerization-inducing mutations was located at a potential protein–protein interface. This work demonstrates that by imposing a selective pressure demanding greater thermostability, mutations can lead to increased oligomerization and stabilization, providing support for the hypothesis that oligomerization is a viable evolutionary strategy for protein stabilization.

Introduction

Oligomerization is one of the most fundamental biophysical interactions in protein chemistry. Bioinformatics and pull-down experiments have revealed that a large number of proteins exist as homo-oligomers consisting of two or more identical chains [1–4]. Similarly, many proteins function in the cell as hetero-oligomers composed of non-identical chains [5–9]. Oligomerization has been shown to play important roles in the genetic economy [10], functional gain [11–13], structural stability [14,15], allosteric regulation [16–19], and protection from degradation [17]. Given the biological importance of oligomerization, there is substantial interest in understanding how the evolution of new oligomeric species occurs [20,21], engineering new oligomeric structure [22,23], and developing drugs targeted at complex assembly and disassembly [24,25].

The cellular milieu is a precarious environment for the evolution of oligomeric proteins as the physical forces that drive beneficial protein association are the same as those that drive deleterious aggregation [26,27]. It is well established that changes in protein sequence through non-synonymous point mutations, insertions, and deletions can shift the balance of oligomeric states [21,28–30]. Both rational mutagenesis and directed evolution have been exploited to advance our understanding of how oligomers form [31–34] and to design new hetero-oligomers [35,36].

Protein flexibility, shape, and symmetry have all been identified as being important for the formation of new oligomeric structure [37,38], since symmetrical and complementary interfaces may form stronger interactions than heterologous surfaces [39–41]. Despite a growing understanding of the nature of oligomers and protein complexes, it is difficult to predict how a new and beneficial protein–protein interface will develop, let alone design one *de novo* [23,42,43]. Moreover, despite intense study, *de novo* evolution of oligomeric structure has not been observed directly, to the best of our knowledge.

The fundamental importance of stability in protein evolution and the stabilizing contribution of oligomerization (*via* reduction of the surface-to-volume ratio of the complex compared to a free monomer) are well established [44,45,2]. Protein–protein interactions can provide stabilizing polar or hydrophobic contacts (although interfaces in homodimers predominantly involve polar interactions), leading to tighter molecular packing and offering protection from denaturation [2,46]. Indirect evidence for the stabilizing effects of protein–protein interactions comes from thermophilic Archaea and bacteria, where oligomerization has been suggested to be one of the contributing factors to the high thermostability of proteins in these organisms [47,48]. Other examples of oligomerization leading to increased stability come from studies in which the disruption of protein–protein interfaces leads to decreased thermostability or enzymatic activity [14,49] or the engineered formation of oligomers leads to increased stability [50,51]. However, despite several lines of evidence that oligomerization increases protein stability, most of the evidence for an evolutionary role for oligomerization had been indirect until the recent work of Perica et al., who used ancestral protein reconstruction to identify specific mutations directly involved in the process [20]. Notably, many of the mutations were found to be remote from the protein–protein interface.

LcαE7 is a carboxylesterase involved in organophosphate insecticide resistance in the sheep blowfly, *Lucilia cuprina* [52]. We have previously reported an experiment in which we performed directed evolution of LcαE7 in order to stabilize the protein for crystallization. However, there was little analysis of the evolution of thermostability, beyond the observation that after four rounds of evolution a variant (D83A, M364L, I419F, A472T, I505T, K530E, and D554G) was obtained that readily crystallized and displayed enhanced thermostability [53]. In this work, we have focused on the process by which the thermostable variant evolved and the nature of the stabilization. Here, we demonstrate that stabilization of the protein occurred *via* two related routes: stabilization of the monomeric protein through improved side-chain packing, and through the enrichment of more stable dimeric and tetrameric species. Oligomerization as a route to thermostability has been inferred through analysis of extant proteins and through ancestral reconstruction [20], but seldom has *de novo* evolution of oligomerization been observed. These results allow us to understand the first steps in this process, which will benefit future engineering efforts.

Results

Directed evolution of LcαE7 for increased thermal stability

As reported previously, the wild-type (WT) LcαE7 protein was unstable both during and after purification [53]. Thus, the original rationale behind the design of this directed evolution experiment was to extend the half-life of LcαE7 at temperatures that it might experience during expression and purification to facilitate crystallization. To achieve this, we designed a medium throughput screen in which LcαE7 was heterologously expressed in *Escherichia coli*, replica plated onto filter paper, and assayed colorimetrically for activity using 2-napthyl acetate and fast-red dye. By randomly mutating the LcαE7 gene in an expression vector and incubating the replica-plated *E. coli* transformed with the mutant library on filter paper for up to 1 h at elevated temperatures, we were able to iteratively select more thermostable variants and progressively increase the thermostability of the protein. After one round of random mutagenesis, only marginal improvements in activity over that of the WT enzyme were evident. Accordingly, these variants were not sequenced, but potentially improved variants were pooled for further random mutagenesis (which includes some recombination [54]) ahead of three further rounds of evolution. By round 2, significant increases in thermostability

Table 1. Mutations incorporated into LcαE7 during laboratory-directed evolution for thermostability

	Round of evolution and mutation						
	2a	2b	2c	2d	3a	3b	4a
Residue							
Asp83							Ala
Ala285			Ser				
Met364							Leu
Ile419				Phe	Phe		Phe
Ala472		Thr	Thr	Thr	Thr	Thr	Thr
Phe478	Leu						
Ile505						Thr	Thr
Lys530		Glu				Glu	Glu
Asp554							Gly

Mutations were not recorded in the first round of mutagenesis. In round 2, four variants (2a, 2b, 2c, and 2d) were selected for DNA shuffling and mutagenesis resulting in round 3 variants. In round 3, two variants (3a and 3b) were selected for DNA shuffling and mutagenesis resulting in the round 4a variant.