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Introduction of a thermophile-sourced ion pair network in the fourth beta/alpha unit of a psychophile-derived triosephosphate isomerase from *Methanococcoides burtonii* significantly increases its kinetic thermal stability

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ABSTRACT

Hyperthermophile proteins commonly have higher numbers of surface ionic interactions than homologous proteins from other domains of life. PfuTIM, a triosephosphate isomerase (TIM) from the hyperthermophile archaeon, *Pyrococcus furiosus*, contains an intricate network of 4 ion pairs in its 4th beta/alpha unit, (β/α)4, whereas MbuTIM, a triosephosphate isomerase from a psychrophile archaeon, *Methanococcoides burtonii*, lacks this network. Notably, (β/α)4 is the first element of the structure formed during folding of certain TIM-type (beta/alpha)8 barrel proteins. Previously, we have shown that elimination of PfuTIM's ion pair network in PfuTIM significantly decreases its kinetic structural stability. Here, we describe the reciprocal experiment in which this ion pair network is introduced into MbuTIM, to produce MutMbuTIM. Recombinant MbuTIM displays multi-state unfolding with apparent Tm values of autonomous structural elements approaching, or above, 70 °C, when a temperature scanning rate of 90 °C/h is used. The protein displays significant intrinsic kinetic stability, i.e., there is a marked temperature scan rate-dependence of the Tm values associated with unfolding transitions. The Tm values drop by as much as ~10 °C when the temperature scanning rate is lowered to 5 °C/h. MutMbuTIM, incorporating PfuTIM's ion pair network, shows significantly higher apparent Tm values (raised by 4–6 °C over those displayed by MbuTIM). MutMbuTIM also displays significantly higher kinetic thermal stability. Thus, it appears that the thermal stability of triosephosphate isomerase can be increased, or decreased, by either enhancing, or reducing, the strength of ion pair interactions stabilizing (β/α)4, presumably through reduced cooperativity (and increased autonomy) in unfolding transitions.

1. Introduction

1.1. Thermodynamic versus kinetic thermal stability

Proteins tend to withstand extreme (physical/chemical) environments that are structurally destabilizing in a manner based mainly upon whether they possess either (or both) of the following properties: (a) high thermodynamic stability, and (b) high kinetic stability. Proteins that are highly thermodynamically stable resist unfolding under extreme conditions essentially because the energy provided by the environment (e.g., high temperature) fails to shift the protein's conformational equilibrium from the native state into the unfolded state. When such proteins fail to display partial or complete unfolding at a given temperature, in theory, they can be relied upon to continue to resist undergoing unfolding at that temperature over infinitely long periods of incubation. Proteins that are not highly thermodynamically stable (but which have a high degree of kinetic stability) can also fail to show unfolding initially, just as is the case with highly thermodynamically-stable proteins; however, such proteins do gradually undergo thermal unfolding over extended periods of incubation at the given temperature. Therefore, in such cases, it is to be concluded that the environment does provide the energy equivalent of the difference in free energy between folded and unfolded states, although unfolding occurs extremely slowly, i.e., the protein displays kinetic stability. The attainment of conformational equilibrium in such 'kinetically stable' proteins from thermophile and hyperthermophile microbes

Abbreviations: MbuTIM, *Methanococcoides burtonii* triosephosphate isomerase; MutMbuTIM, Mutant *Methanococcoides burtonii* triosephosphate isomerase; PfuTIM, *Pyrococcus furiosus* triosephosphate isomerase; MutPfuTIM, Mutant *Pyrococcus furiosus* triosephosphate isomerase; MALDI-TOF, Matrix-assisted laser desorption-ionization time-of-flight; MRE, Mean residue ellipticity; T_m, Temperature of melting; CD, Circular dichroism; DSC, Differential scanning calorimetry

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appears to have become offset to such a high degree that populations of such proteins retain structure, and function, for inordinately long lengths of time [extending to years, or even centuries of time] under conditions that eventually prove to have been structurally destabilizing from a purely thermodynamic viewpoint. Numerous instances are now known of proteins that unfold inordinately slowly, even though the free energy change associated with their unfolding is only 5–15 kcal/mol [1–4]. It may be only a matter of time before the known numbers of such proteins increases to much larger numbers, since more and more proteins from thermophiles and hyperthermophiles are being produced and studied. It is impossible to overemphasize the point that high kinetic thermal stability is not true stability in thermodynamic terms. It is merely an 'operational' or 'functional' stability, since its existence allows proteins to retain structure(s) and function(s) for long durations of exposure to environmental conditions that eventually cause unfolding [5–10].

From a purely functional point of view, however, high kinetic stability must be thought to be at least as important as (if not even more important than) high thermodynamic stability, since naturally-occurring proteins are mostly required to survive and function for a limited duration of a few minutes or hours [11] and not for ever. If a protein is folded into a sufficiently kinetically-stable structure, it can resist unfolding and retain full functionality for the entire duration of its normal/designated existence *in vivo* without needing to either: (a) be highly thermodynamically stable, or (b) even be folded into the most stable of all structures conceivable for that protein. Therefore, understanding the basis of kinetic stability in proteins is critical to the development of applications allowing proteins to be stabilized or destabilized by design using rational protein engineering-based methods [12–15].

1.2. The kinetic stability of proteins from extremophile organisms

Proteins from extremophile organisms tend to be both thermodynamically as well as highly kinetically stable. Increasingly, it appears that it is the high kinetic stability of such proteins that is primarily responsible for their inordinately high structural stability. Measurements of the thermodynamic stabilities of hyperthermophile proteins at their normal temperatures of functioning, under conditions of conformational equilibrium, suggest that the free energy changes associated with their unfolding at 'hyperthermophile' temperatures are only marginally higher than that of analogous mesophile homologs at 'mesophile' temperatures [1–4]. More specifically, if the change associated with unfolding is not of the order of ~5–15 kcal/mol (as it tends to be for mesophile proteins), it tends to be somewhat higher, at 20–25 kcal/mol, but generally no higher. In contrast, the kinetic stabilities of mesophile and hyperthermophile homologs are very different. For conditions that initiate unfolding, it is well-known that unfolding rates are dependent on temperature, or denaturant concentration, varying over one or two orders of magnitude [16,17]. In contrast, thermophile proteins are generally found to attain conformational equilibrium over timescales that are several orders of magnitude longer than those applicable to unfolding of mesophile homologs [5–10,18].

1.3. A hypothesis concerning kinetic thermal stability

For several years now, our group has been interested in understanding and manipulating kinetic stability in proteins. Our contention is that a protein's kinetic thermal stability depends mainly on the autonomy of structural stabilization of component sub-structures (e.g. helices, sheets, or combinations of such structures, forming supersecondary structural elements) within the protein, with the number and nature of such sub-structures determining the level of cooperativity that is seen in the overall (global) unfolding process for the entire protein's structure [19–25]. More specifically, our contention is that if a protein's sub-structures tend to be reasonably autonomously stable, they would not be expected to be dependent on the energy of stabilization gained through packing interactions with neighboring sub-structures for formation (or retention) of structure. Consequently, structural unfolding would tend to be non-cooperative at the global level. In other words, if a particular sub-structure in a hyperthermophile protein were to undergo unfolding, this would have little effect on neighboring sub-structures. Therefore, no cooperative global unfolding of the protein's structure would occur as a consequence of the unfolding of any individual sub-structure. The non-occurrence of a cooperative global unfolding transition would afford each particular sub-structure undergoing unfolding the opportunity to refold and re-associate with its neighboring sub-structure(s). Thus, each unfolding event involving a sub-structure would be unproductive in respect of its influence on global unfolding, frustrating the unfolding process and effectively slowing it down. Our hypotheses, therefore, is that reduced cooperativity of global unfolding (achieved through increased autonomy of formation, and stabilization, of sub-structures) is one mechanism by which a protein can gain high kinetic stability, regardless of the exact details of the mechanisms by which individual sub-structures are stabilized.

1.4. Protein engineering of kinetic thermal stability in a TIM Barrel protein

In a recent paper [25], we have shown that mutational disruption of an elaborate ion pair network on the surface of a small element of sub-structure in *Pyrococcus furiosus* triosephosphate isomerase (PfuTIM) achieves a dramatic reduction in the protein's kinetic thermal (conformational) stability, causing it to transform from being an extraordinarily hyperthermostable protein into a protein of ordinary thermostability. In this particular case, the element of sub-structure happened to be a helix in the fourth beta/alpha unit, $(\beta/\alpha)_4$, of the eightfold beta/alpha barrel (TIM barrel) structure of PfuTIM. Notably, the first four β/α units and, in particular, the fourth β/α unit, $(\beta/\alpha)_4$, is thought to play a crucial role in the folding of proteins with the TIM barrel type of fold, with $(\beta/\alpha)_4$ being the first of the β/α units to form during folding [26,27]. The precise mutations that we introduced (Fig. 1A and B) were based on an examination of the analogous stretch of sequence in a related triosephosphate isomerase from a psychrophile organism, *Methanococcoides burtonii* (MbuTIM). In MbuTIM, no scope exists for the occurrence of analogous ion pair interactions within the corresponding helix. The entire sequences of the two proteins are aligned and shown in Supplementary Fig. 1.

1.5. The scope of the present work

In the present paper, our intention was initially to perform an experiment constituting the exact reverse of the previous experiment, i.e., we wished to introduce PfuTIM's ion pair network into MbuTIM to see whether this would dramatically improve the kinetic stability of this psychrophile/psychrotolerant protein. While there was indeed a significant enhancement of kinetic thermal stability in MbuTIM as a consequence of this engineering, we were surprised to discover that MbuTIM was naturally a reasonably intrinsically kinetically stable protein in its own right. Thus, rather than introducing some kinetic thermal stability into a protein that we had presumed would lack substantial kinetic stability a priori, we found MbuTIM to be substantially kinetically stable; however, we managed to significantly further improve both MbuTIM's kinetic stability and its thermodynamic stability by introducing PfuTIM's ion pair network into it.

1.6. The organism Methanococcoides burtonii and its proteins

A few words of introduction to the organism, *M. burtonii*, would be in order. *M. burtonii* is a cold-adapted archaeon from the methane-saturated, permanently-cold waters of a lake in Antarctica [28]. It grows naturally at a temperature of about 1 °C, but has a temperature range of growth of −2.5 to 28.0 °C, with optimum growth at 23.0 °C [29]. Recent work done to examine the mechanism of cold adaptation in this organism, through extensive studies of differences in lipid composition and