

Functional modulation of a protein folding landscape via side-chain distortion

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Ultra-high-resolution (<1.0 Å) structures have revealed unprecedented and unexpected details of molecular geometry, such as the deformation of aromatic rings from planarity. However, the functional utility of such energetically costly strain is unknown. The 0.83 Å structure of α -lytic protease (α LP) indicated that residues surrounding a conserved Phe side-chain dictate a rotamer which results in a $\sim 6^\circ$ distortion along the side-chain, estimated to cost 4 kcal/mol. By contrast, in the closely related protease *Streptomyces griseus* Protease B (SGPB), the equivalent Phe adopts a different rotamer and is undistorted. Here, we report that the α LP Phe side-chain distortion is both functional and conserved in proteases with large pro regions. Sequence analysis of the α LP serine protease family reveals a bifurcation separating those sequences expected to induce distortion and those that would not, which correlates with the extent of kinetic stability. Structural and folding kinetics analyses of family members suggest that distortion of this side-chain plays a role in increasing kinetic stability within the α LP family members that use a large Pro region. Additionally, structural and kinetic folding studies of mutants demonstrate that strain alters the folding free energy landscape by destabilizing the transition state (TS) relative to the native state (N). Although side-chain distortion comes at a cost of foldability, it suppresses the rate of unfolding, thereby enhancing kinetic stability and increasing protein longevity under harsh extracellular conditions. This ability of a structural distortion to enhance function is unlikely to be unique to α LP family members and may be relevant in other proteins exhibiting side-chain distortions.

protein distortion | protein structure | X-ray crystallography | protein stability | protein lifetime

The physicochemical forces that determine macromolecular structure and function are generally thought to be well understood. The predominant forces include well-studied hydrogen bonding, van der Waals, and ionic interactions. By contrast, bonded terms are largely ignored as it is commonly held that bond lengths and angles are at or very close to canonical values in the native state. This view is so widely adopted that nearly all crystallographic and NMR structure determination methods include restraints to optimal values. However, distortion of covalent bonds in protein sidechains away from their low energy configurations has now been noted in several ultrahigh-resolution structures (1, 2) (Fig. 1*A*), but no functional role for the distortion has been ascribed. We examine the functional consequences of side-chain distortion in α -lytic protease (α LP), in which a 0.83 Å structure (2) revealed a surprisingly large distortion of the aromatic ring of a conserved Phe (Phe228) by almost 6° from planarity (Fig. 1*B*; about 2.6 standard deviations from the mean in Fig. 1*A*).

The α LP sub-family of serine proteases is one of the best-studied examples of a kinetically stable protein (3, 4); i.e. a protein that retains its native state through exceedingly slow unfolding rates. The folding landscape of α LP is distinct from typical, thermodynamically stable proteins in that its folding barrier is very high (corresponding to a $t_{1/2} \sim 1,800$ y), and the native state (N) is less stable than the unfolded forms by an unprecedented 4 kcal/mol (5). Therefore, α LP's functional stability is dictated solely by its extremely high unfolding barrier [$t_{1/2} \sim 1.2$ y for

N to the transition state (TS)] rather than the overall free energy difference between its native and unfolded states (5). In α LP and other family members, kinetic stability is a preferred mechanism for stability because the high unfolding energy barrier and the reduced native state dynamics result in enhanced longevity for these proteases in harsh environments, such as pH fluctuations and high protease concentrations (6–9).

A covalently attached folding catalyst (the pro region) performs two crucial functions to facilitate α LP folding (5): It (*i*) accelerates folding by nine orders of magnitude so that folding can occur on a biologically relevant timescale and (*ii*) stabilizes the native state over the unfolded state so that the reaction is pulled in the direction of the native state. The pro region is subsequently degraded, leaving the native protease in its metastable but kinetically trapped active form (10).

Based on thermodynamic, kinetic, mutational, and computational studies of α LP and its homologs (6, 8, 11–13), our group has proposed a TS structure wherein each β -barrel domain is relatively well folded but the interface between the two domains is not fully formed. Although these studies have provided a phenomenological model for the TS, an explanation for the source of the unique kinetic stability observed in α LP is still lacking. Specifically, what causes the TS to be at such a high energy and why is the N destabilized relative to the unfolded forms? Given the context, the observed distortion of Phe228 led to the suggestion that it might somehow play a role in α LP kinetic stability; perhaps by suppressing native state dynamics (2), but this hypothesis had not been experimentally tested.

By analyzing mutants of α LP as well as related family members with altered folding landscapes, we show that distortion of Phe228 correlates with the degree of kinetic stability and contributes directly to the height of the unfolding barrier by preferentially destabilizing TS. However, contrary to previous hypotheses (2), the distortion appears to not play a role in the ultracooperativity observed in α LP unfolding. This represents, to our knowledge, a previously uncharacterized functional role for side-chain distortion. Finally, our meta-analysis of previous ultrahigh-resolution structures indicates that side-chain distortion is a surprisingly common and underappreciated phenomenon that may be playing functional roles in other protein families as well.

Results and Discussion

We used the wealth of information in sequence data to understand the structural basis for kinetic stability in the secreted bacterial

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Data deposition: Atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 3URC, 3URD, and 3URE).

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is used to suppress native state dynamics in α LP, leading to its ultracooperative unfolding (7). To assess whether side-chain distortion suppresses α LP native state dynamics, we measured proteolysis using an autolysis assay in which the unfolding rate (measured by tryptophan fluorescence) and the loss of activity are compared across a large α LP concentration range. Proteolysis has been used as a very effective probe for measuring subglobal unfolding transitions (19–21). It has been previously established that the autolysis rate for α LP closely matches that of the global unfolding rate, indicating that autolysis is limited by global unfolding and is not due to partially unfolded forms (7), in contrast to many thermodynamically stable proteins (19, 20). If nonglobal unfolding events are populated to an appreciable degree, then the autolysis rate will be faster than the global unfolding rate. Because α LP is both an enzyme and substrate in the autolysis reaction, the autolysis rate should be especially sensitive to changes in concentration ($k_{\text{autolysis}}$ is roughly proportional to $[\alpha\text{LP}]^2$.) In addition, increasing the pH should further increase proteolytic activity by activating key catalytic residues.

We measured the rate of protease inactivation by loss of activity and/or fluorescence at 0.05 μM and 5 μM Repack- α LP at pH 8.0, close to α LP's maximal protease activity, thus increasing the potential rate of autolysis up to $\sim 10^4$ -fold. However, the protease inactivation rate at high concentration matched the unfolding rate at low α LP concentration as measured by loss of tryptophan fluorescence (Fig. S5). This is an extremely robust result as the two rates are equivalent over a wide range of denaturant concentration and pH. Thus, removal of Phe228 distortion has no impact on the degree of unfolding cooperativity as measured by autolysis rates, indicating that Phe228 does not play a functionally important role in the suppression of native state dynamics in α LP as previously hypothesized. However, the inactivation and unfolding rates for the mutants are significantly increased relative to WT- α LP, demonstrating that side-chain distortion does play a significant role in increasing the functional lifetime of α LP via increasing the unfolding energy barrier.

The T181A to Gly and Repack mutations were designed as two independent strategies for alleviating strain in Phe228. In addition to the dominant effect of relieving side-chain strain, both of these mutants also lead to subtle backbone improvements in the β -sheet around Phe228 (three cross-strand H-bonds are ~ 0.1 Å shorter). These mutations produce coincident and unexpected thermodynamic results: the stabilization of N and TS. Because the common feature is the reduction of strain, the implication is that strain destabilizes TS and N in WT- α LP. The greater effect seen in Repack most likely results from better packing using the evolutionarily optimized SGPB core and the avoidance of the entropic consequences of the Gly mutation.

Because these mutations affect the stability of both TS and N, it is useful to quantify what fraction of the energetic effect has direct functional consequences. The relative contribution of strain to folding kinetics versus its contribution to thermodynamic stability can be quantified by Φ -value analysis ($\Phi = \Delta\Delta G^\ddagger / \Delta\Delta G$) (22). Φ values typically range from 0 to 1.0, and are interpreted as follows: Interactions altered by mutation have no contribution to the TS ($\Phi = 0$) or fully contribute to the TS ($\Phi = 1$), with fractional Φ values indicating a partial involvement in the TS structure. The Φ values for strain removal in both T181A to Gly and Repack mutants are remarkable: 1.9 (± 0.5) and 2.7 (± 0.5), respectively. Φ values are usually reported without their associated errors, but can be very susceptible to errors when the difference between $\Delta\Delta G^\ddagger$ and $\Delta\Delta G$ is small (23). Because our Φ values are extremely noncanonical, we propagated the errors to ensure these values are not an artifact of imprecise measurements, as interpretation of Φ values of this magnitude is not without controversy (24–26). Although not described in this context previously, such noncanonical Φ values should be a hallmark of removing interactions that contribute most significantly to kinetic

stability, as they indicate interactions that affect TS more than N. Values such as these also suggest a TS structure that is highly organized, at least in the vicinity of the mutations.

The data from both Repack and the Thr181 mutations provide a consistent picture of the role of strain, confirming three key features of our hypothesis: (i) strain from the Phe228 distortion is indeed present in N, (ii) this strain also manifests itself in the TS, and (iii) the strain energy in TS must be ~ 2 – 3 -fold greater than in N, as indicated by the Φ values (Fig. 4A). The excess contribution of side-chain distortion to TS directly indicates that the three-dimensional environment of Phe228 must be highly structured in the TS, although whether the degree of mechanical distortion is enhanced or if there are simply fewer compensatory interactions in the TS is not known. If the increased strain in the TS occurs entirely through deformation of the Phe, then we estimate the distortion of Phe228 (modeled as a Hookean spring) in the TS must be between 8 – 10° . This extends previous insights into the structure of the TS, which had only implicated regions around the domain interface as being important (6, 8). More importantly, the enhanced contribution of side-chain distortion to the TS provides a critical rationale for incorporating strain: the increased energetic differential between TS and N directly results in an increased kinetic stability (Fig. 4A), which, in turn, leads to a significantly extended functional lifetime.

Our results can rationalize important issues of pro-dependent folding of α LP and its homologs (4). The structure of α LP bound by its pro region showed that the pro region is a two-domain C-shaped protein that binds to and surrounds the α LP C-terminal

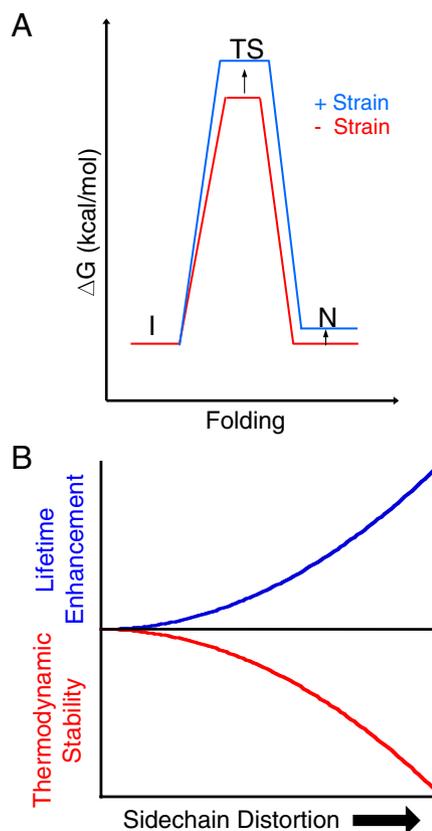


Fig. 4. Strain increases kinetic stability. (A) A schematic model for the effect of strain on the folding landscape of the kinetically stable proteases. Strain destabilizes the Transition State (TS) more so than the native state (N), thereby enlarging the unfolding free energy barrier and extending protease lifetime. (B) Schematic illustrating the differential effects of strain on kinetic versus thermodynamic stability. The energetic trade-off (modeled as a Hookean spring) between kinetic and thermodynamic stability is shown as found in α LP.

domain (27). Based on these results, it was hypothesized that much of the folding defect of α LP would be localized to the C-terminal domain. In this work, we localize a significant folding defect to the distortion of Phe228. Further, our data suggests that the C-terminal domain is highly structured in the TS, which is corroborated by the tight binding of the pro region to both TS and N (5). These data suggest significant structural similarity between TS and N. Therefore, we propose that the role of the large pro region of α LP and its close homologs is to clamp onto and organize the C-terminal domain such that the distortion of Phe228 is stabilized.

A key advantage of the kinetically stable proteases compared to their thermodynamically stable homologs is their increased ability to survive in harsh environments (7). The data presented here indicate that one key component in the evolution of kinetic stability is the introduction of geometric strain into the TS via the distortion of planar, aromatic side-chains, resulting in a dramatic enhancement of functional lifetime. However, the cost of introducing strain is significant; the additional improvement in lifetime comes at an ever-increasing penalty in terms of foldability and N stability (Fig. 4B). Although the substantial distortion observed in N (Fig. 1B) can be viewed as wasted residual strain from the TS, this strategy is surprisingly efficient: 2- to 3-fold higher strain energy is realized in the TS compared to N. This compares quite favorably to the fractional contribution to kinetic stability (Φ values between 0 and 1) that would be imparted by a typical thermodynamically stabilizing interaction such as an H-bond or van der Waals interaction.

For more conventional thermodynamically stable proteins, introduction of side-chain distortion would cause significant destabilization (Fig. 4B), thus we expected such distortions to be relatively rare in proteins. Surprisingly, $\sim 20\%$ of all proteins in our dataset of ultrahigh-resolution structures display a Phe or Tyr residue with a distortion similar to or even greater than that found in α LP (Fig. 5). Thus, the functional benefits must outweigh the energetic penalties associated with these distortions, suggesting that other proteins may also use the strain energy from side-chain distortions to modulate biological activity. In the case of α LP and its homologs, strain energy is used to extend functional lifetime, but we envision side-chain distortion also being relevant for other important macromolecular functions, such as allostery and catalysis. Although the functional importance of distortion in enzyme substrates (28–30) and cofactors (31–34) has long been appreciated, and recent analysis points to conserved

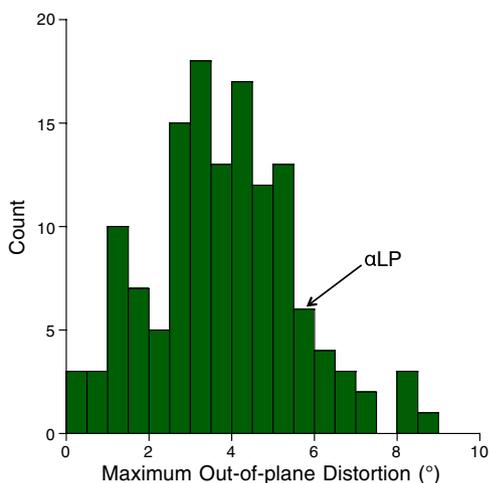


Fig. 5. Large distortions are not unique to α LP. Shown is a histogram of the largest Phe or Tyr distortion per protein from the ultrahigh resolution structures in our dataset. Approximately 20% of the proteins contained at least one Phe or Tyr residue with a distortion comparable or even larger than that of Phe228 in α LP.

peptide bond distortions (35), the observation of side-chain distortion and the discovery that it can play a significant role in modulating energetic landscapes to provide biologically important advantages is quite unique. This study identifies an unanticipated challenge: the need to observe structurally subtle yet functionally significant covalent distortions to fully understand the energetic forces acting on proteins and their impact on function.

Materials and Methods

Database of Phe Distortions. A database of atomic resolution structures (<1.0 Å) was built by examining the Protein Data Bank (PDB, www.pdb.org). A size threshold of 30 residues was enforced and structures with $>90\%$ sequence identity to another previously accepted member of the database were not included. Phenylalanine residues with multiple conformations were not included. The C_{β} - C_{γ} - C_{ζ} angle in Phe is an imperfect measure of out-of-plane ring distortion because in-plane ring distortions can also reduce the angle from its idealized value of 180° . To eliminate the effect of in-plane distortions, we developed the following protocol. For each Phe or Tyr, the side-chain is translated and rotated such that C_{β} is at the origin, C_{γ} is on the positive z axis, $C_{\delta_1}(x) = C_{\delta_2}(x)$, and $C_{\delta_1}(y) < C_{\delta_2}(y)$. The C_{β} - C_{γ} - C_{ζ} angle is unaffected by this transformation and measures the uncorrected distortion. Then $C_{\zeta}(y)$ is set equal to 0, eliminating the sideways bend, as all out-of-plane distortion comes from the value of $C_{\zeta}(x)$. The new C_{β} - C_{γ} - C_{ζ} angle measures the corrected distortion. In addition, the direction of the distortion can be defined because C_{δ_1} and C_{δ_2} are consistently oriented. Here, the bend angle is defined by the corrected C_{β} - C_{γ} - C_{ζ} if $C_{\zeta}(x) < 0$, and $360 - C_{\beta}$ - C_{γ} - C_{ζ} if $C_{\zeta}(x) > 0$. If C_{δ_1} and C_{δ_2} are assigned randomly when the crystal structure is solved, the distribution of bend angles should center at 180° , which we find to be the case. Side-chain distortion angles from structures with multiple protein copies per asymmetric unit are averaged values.

Sequence Analysis. From a BLAST search of the National Center for Biotechnology Information database using the α LP sequence as query, 47 homologous protease sequences were retrieved. The sequences were aligned with ClustalW and a phylogenetic tree constructed using MacVector. Proteases were categorized based on sequence covariation patterns expected to determine the rotamer of Phe228 (2).

Cloning/Protein Production. α LP mutants were made using Quik-Change Site-Directed mutagenesis (Stratagene), and protein was expressed and purified according to published protocols (36).

Folding/Unfolding. Uncatalyzed folding of α LP was performed as described in Sohl et al. (5), except that the total protein concentration was ~ 4 μ M and the assay was calibrated within each timepoint using standards of no protein and 25 pM WT- α LP to improve the precision of each measurement. Unfolding was measured by loss of tryptophan fluorescence (excitation 283 nm, emission 320 nm) as described (13). The unfolding rate of α LP at 0° C was determined by extrapolation from data of Jaswal et al. (13). The denaturant binding model was used to model the unfolding behavior of the Repack and T181A mutants because it provides an accurate empirical fit of the curved data. Its use has been validated for WT- α LP, where it was established that the observed curvature was not due to transition state movement (13), but rather due to electrostatic effects from the ionic denaturant guanidine, as linear extrapolations from the nonionic denaturant urea yield the same rate constant (37). It is unknown why certain mutants have curvature in their unfolding denaturant titration, but it has been seen in multiple cases. Rate constants were converted to ΔG using standard transition state theory. Although the appropriate "preexponential factor" for an unfolding or folding reaction is controversial, $\Delta\Delta G^{\ddagger}$ is unaffected by the choice of a preexponential term.

Autolysis assays were performed at 0° C and pH 8.0 (10 mM Tris). Unfolding was measured at 50 nM protein using intrinsic tryptophan fluorescence (283 nm, 322 nm). Inactivation at 5 μ M was measured by loss of proteolytic activity toward the synthetic substrate Succinate-Ala-Pro-Ala-paranitroanilide as previously described (7) and by tryptophan fluorescence.

Crystallization/Structural Analysis. The crystallization of T181A and T181G α LP mutants was performed as described for WT- α LP (2). Crystals were in space group P3₂21 with one molecule per asymmetric unit. However, the Repack mutant could only be crystallized under slightly different conditions (addition of 10 mM CuCl₂) and in a different space group P6₁22 with two α LP molecules per asymmetric unit. Diffraction data were collected at the Advanced Light Source, Beamline 8.2.2, and processed in HKL2000 (38). For the Repack

mutant, the structure was solved using Molecular Replacement with WT- α LP (with residues 181, 199, 210, and 228 as alanine) as a starting model. The T181A and T181G mutants were solved with a starting model of WT- α LP (2) with residues 228 and 181 as alanine or glycine, depending on the mutant. Initial refinement was performed in CNS (39), and anisotropic B-factors were modeled using REFMAC (40). Structural alignments were made using Combinatorial Extension (41).

The distortion of Phe228 in TFPA has been described previously (6). The 1.85 Å structure of NAPase was reported previously (8), in which distortion of Tyr228 in NAPase could be detected even with planarity restraints during refinement (~4.2°). For this work, planarity restraints were removed in CNS

(39), and the distortion in the two copies of NAPase in the asymmetric unit were averaged together. Distortion of Phe 228 in SGPB was measured using PDB ID code 2QAA (15).

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