Mechanical unfolding of acylphosphatase studied by single-molecule force spectroscopy and MD simulations

Gali Arad-Haase[†1], Silvia G. Chuartzman[†1], Shlomi Dagan[†], Reinat Nevo[†], Maksim Kouza[#], Mai Khanh Binh[++], Nguyen Tien Hung[++], Mai Suan Li[‡*], and Ziv Reich[†*]

[†]Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel

[#]Department of Physics, Michigan Technological University, Houghton, MI 49931, USA

[++]Saigon Institute for Computational Science and Technology, 6 Quarter, Linh Trung Ward, Thu Duc District, Ho Chi Minh City, Vietnam

[‡]Institute of Physics, Polish Academy of Sciences, Al. Lotnikow 32/46, 02-668 Warsaw, Poland

1 *These authors contributed equally to the work*

***Correspondence: ziv.reich@weizmann.ac.il and masli@ifpan.edu.pl**

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ABSTRACT

Single-molecule manipulation methods provide a powerful means to study protein transitions. Here we combined single-molecule force spectroscopy and steered molecular dynamics simulations to study the mechanical properties and the unfolding behavior of the small enzyme acylphosphatase (AcP). Mechanical unfolding of AcP occurs at relatively low forces in an all-or-none fashion and is decelerated in the presence of a ligand, as observed in solution measurements. The prominent energy barrier for the transition is separated from the native state by a distance that is unusually long for α/β proteins. Unfolding is initiated at the C-terminal strand (β_T) that lies at one edge of the β-sheet of AcP, followed by unraveling of the strand located at the other. The central strand of the sheet and the two helices in the protein unfold last. Ligand binding counteracts unfolding through stabilization of contacts between an arginine residue (Arg23) and the catalytic loop and β_T of AcP, which renders the force-bearing units of the protein resistant to force. This stabilizing effect may also account for the decelerated unfolding of ligand-bound AcP in the absence of force.

INTRODUCTION

Understanding the determinants of the native structure of proteins and the way this structure is gained or lost during folding and unfolding are central objectives in structural biology. During the last decade, the arsenal of techniques available for studying these issues has been complemented by the use of the atomic force microscope (AFM) and optical tweezers to induce and record the unfolding of single protein molecules through the application of a stretching force, as well as to follow their folding characteristics, in the presence or absence of force (1-8). Compared to traditional bulk assays of protein folding/unfolding, such pulling experiments have several distinguishing attributes: (i) they provide a direct measure of the molecules' mechanical stability with sensitivity sufficient to detect purely entropic-driven forces (ii) they act solely on the molecules of interest, leaving the environment unaffected and (iii) they are performed at the single-molecule level, enabling to obtain information that is often masked by ensemble averaging. Furthermore, in contrast to chemical or thermal-induced denaturation where the reaction coordinate is generally unknown, the application of mechanical force sets a relatively well-defined reaction coordinate (*i.e.,* end-to-end distance of the polymer chain). This simplifies data interpretation and allows for direct comparison with results obtained from molecular dynamics (MD) pulling simulations (9, 10), enabling to acquire a detailed, sometimes atomistic, description of the process investigated.

Difficulties arising from spurious interactions between the AFM tip and the substrate-sample surface as well as from the random nature of the interaction between the AFM tip and the molecules under study usually preclude analysis of small monomeric proteins by force spectroscopy. Earlier studies therefore concentrated on proteins or segments derived from proteins that naturally occur as tandem arrays of globular modules, such as titin (11), tenascin (12), spectrin (13), and fibronectin (14). Such proteins frequently operate under tensile stress and, thus, have evolved to withstand mechanical deformations. Naturally occurring modular proteins usually contain a heterogeneous set of domains that vary in structure and stability. It is thus generally not possible to assign observable events to individual modules. In addition, the exact 3D structure of the domains present in these proteins is often unknown and mutants are not readily available. The introduction of recombinant (15, 16) or chemically linked (17-19) polyproteins - polymeric protein constructs composed of repeated domains derived from, in principle, any protein, extended the studies to individual protein modules and to proteins that have not been selected to resist mechanical forces (not discussed here, pulling experiments can also be performed on membrane proteins (4)). The availability of forced unfolding data on well-characterized protein domains has enabled the use of mutants and MD simulations and has facilitated comparison between mechanical- and chemical- or thermal-induced unfolding. The results obtained from these experiments and from simulations have provided important information about the mechanical stability of proteins and its relation to protein structure and function. They have also provided valuable insights into protein unfolding/refolding dynamics as well as into features of the free energy landscapes that underlie forced unfolding and, in some cases, into the pathways explored during this process.

Acylphosphatase (AcP; E.C. 3.6.1.7) is a small (~100 aa), basic, protein that catalyses the hydrolysis of the carboxyl-phosphate bond present in a diverse set of biological and synthetic compounds (20). In vertebrate, it is found as two isoforms, coined muscle- and common-type AcP (mAcP and ctAcP), which share over 50% sequence homology. In both forms, as well as in

all other orthologs thus far characterized, including bacterial and archaeal, its structure consists of two parallel α-helices packed against a five-stranded antiparallel β-sheet that follows a 4-1-3-2-5(β_T) strand topology (Fig. 1) (21-28). The small size of AcP, its simple (though rather uncommon) topology, and the fact that it lacks intramolecular disulfide bridges (with the exception of some bacterial homologues) or prosthetic groups, make it an attractive candidate for structural and kinetic analyses. Indeed, mAcP and ctAcP and, to a lesser extent, their invertebrate and bacterial and archaeal homologues, have been the subject of many such analyses and their folding and unfolding dynamics are extremely well characterized (see *e.g.,* (23, 26, 29-32)). The structure of their transition state ensembles has likewise been thoroughly investigated, both experimentally (31, 33-35) and by coarse-grained Monte Carlo sampling (36) and all-atom MD simulations (37), using experimentally determined ϕ-values as restraints. The two isoenzymes fold with a two-state kinetics (excluding a *cis-trans* prolyl isomerization phase) under a wide range of conditions, but typically unusually slow. In fact, the human muscular form of AcP (hmAcP) is the slowest autonomous two-state folder known, completing its folding in about four seconds (32). Notably, under certain destabilizing conditions, hmAcP aggregates and subsequently forms amyloid fibrils similar to those found in protein deposition diseases (38-42). The catalytic activity of AcP has also been studied fairly broadly and is relatively well understood (20, 27).

In this work, we combined single-molecule force spectroscopy with Go-type and all-atom SMD simulations to study the mechanical unfolding of the well-characterized form of AcP, hmAcP. The results obtained from the experiments and simulations provide detailed information about the unfolding of AcP under applied force as well as on the countering of this process by ligand binding. They are also used to address general issues pertaining to the correlation between the mechanical resistance of proteins and their conformational stability and secondary structure content and topology and how well results obtained from pulling experiments of polyproteins compare to those derived from traditional solution assays of isolated modules.

MATERIALS AND METHODS

This section is described in the Supporting Material.

RESULTS AND DISCUSSION

Concatenation of AcP does not lead to changes in structure or activity

To study the mechanical unfolding of AcP, we constructed a polyprotein, $[AcP]_4$ (Fig. S1), by concatenating the gene encoding for the C21S variant of human muscle acylphosphatase (32). This variant is commonly used in studies of hmAcP to eliminate complexities associated with the presence of a free cysteine residue and is referred to as AcP throughout the text. As observed by others (43), expression of the polymeric construct proved to be difficult and required the use of a poor growth medium (M9 minimal broth) before induction of gene expression (see Supporting Material). This was likely necessary to prevent residual expression of the polyprotein during the growth phase of the bacteria, suggesting that it was toxic to the cells.

We then tested if the modules present in the concatameric construct preserve the structure and activity of the isolated protein, as these may be altered by inter-domain interactions or by constraints imposed by the linkers that separate the modules in the polyprotein. To examine if the modules constituting $[AcP]_4$ retain the structure of the native protein, we subjected the innate and oligomeric forms of AcP to far-UV CD analysis (Fig. 2 *A*). These, and all subsequent measurements described in this work were performed in 50 mM acetate buffer, pH 5.5, which is optimal for AcP activity and is conventionally used in thermodynamic and kinetic analyses of this protein. Isolated AcP exhibited a CD spectrum characterized by a broad, flat trough between 240 and 210 nm and a positive band centering at 198 nm, primarily reflecting the weighted contributions of its two major secondary structural motifs (~20% helices and ~40% β-strands). The spectrum of $[AcP]_4$ superimposed that of the isolated protein over most of the wavelength range, deviating only in the positive CD band, where the amplitude, but not the shape or position of the band, was higher. This increase in amplitude most likely reflects contributions arising from the additional residues present in the three linker regions that separate the individual domains in the polymeric construct.

Next, we checked whether the individual domains in the polymer preserve the catalytic activity of the innate protein. This was done by following the change in absorbance of 283-nm light during hydrolysis of benzoyl phosphate (44), an AcP substrate. As shown in Fig. 2 *B*, the rate of hydrolysis of the substrate by the monomeric and oligomeric forms of the enzyme (used in equal monomer concentrations) was practically identical.

AcP has relatively low resistance to mechanical force

In addition to the inherent mechanical properties of the molecule under investigation, the measured unfolding forces in force spectroscopy experiments depend on a number of factors. These include the pulling speed, pulling geometry (the relation between the force vector and breakpoint topology), the number of domains in the polyprotein, and the length and composition of the intervening linkers. Nevertheless, data obtained on different proteins and protein domains as well as results derived from SMD simulations enabled drawing some general conclusions on the relationship between protein structure and resistance to applied forces ((2, 3, 5, 6, 18, 45-49), and references therein). It appears that, for proteins extended by their termini, there exists a hierarchy of resistance to mechanical deformations, which is determined predominantly (though

not exclusively) by the content and pattern of the hydrogen bond arrays present in the force-bearing regions of the protein. The most resistant are β-sheet-containing proteins in which the terminal strands are parallel, backbone hydrogen-bonded, and oriented orthogonally to the applied force (forming a so-called 'shear topology'), as seen in titin I27 (11), FNIII (14), ubiquitin (50, 51), GB1 (52), and protein L ((45), see also Table 1 in this article). Also highly stable are β-sheet configurations in which the force-bearing strands are flanked in space by neighboring strands, as exhibited by the designed protein Top7 (48) and which are also present in the aforementioned mechanically stable proteins. In most of these proteins, the flanking strands are connected to the force-bearing strands through β–hairpins, further enhancing mechanical stability. At the other end of the spectrum lie unstructured and β-spiral proteins (*e.g.* elastin (53)) and, moderately more stable, all α-helical proteins in which hydrogen bonding is confined to within individual secondary structures. Stability is therefore dominated by hydrophobic contacts between helices, offering lower resistance to mechanical deformations. Proteins possessing other topologies usually exhibit mechanical stability that lies in between those of the above two classes. As can be seen in Fig. 1, the N and C-terminal β-strands of AcP, which constitute its force-bearing units, are not hydrogen bonded to each other nor are they stabilized by hairpin loops, which are mostly lacking in AcP due to its $\beta\alpha\beta\beta\alpha\beta$ topology. In addition, one of these strands, the C-terminal strand (β_T), is very short and is connected to the β-sheet core through a few hydrogen bonds (Fig. 1). AcP is therefore expected to have only a moderate resistance to tensile forces.

To test the aforementioned prediction, we mechanically unfolded $[AcP]_4$ over a broad range of extension rates, varying from ~30 to 10,000 nm/s. Stretching of the polymer gave rise to a characteristic saw-tooth pattern (Fig. 3 *A*) exhibiting more or less regularly spaced force peaks of varying amplitudes. The rising part of the peaks, which corresponds to the entropic elasticity of the unfolded protein domains, fitted well to a worm-like chain (WLC) model of polymer elasticity, using a persistence length of 0.36 nm. The increment in contour length upon domain unraveling obtained from the fits (which predict this parameter at infinite force) was 33 ± 2 nm (Fig. 3 *A*, inset). This value is practically identical to the one predicted for a fully extended AcP module and expectedly larger than the observed distances between adjacent peaks in the force-extension curves (30 ± 2), indicating that unfolding occurs before the polypeptide chain is fully stretched. The distributions of the most probable unfolding forces recorded at different pulling speeds are shown in Fig. 3 *B*. As can be seen, the unfolding force is parameterized by the rate of extension, being shifted to higher values as the latter increases. Such a shift is expected when the loading rate (df/dt) exceeds the characteristic (spontaneous) time scale of the transition (54), which is clearly the case for AcP. Analysis of the force-extension curves and of the distributions of unfolding forces, as well as the observed increments in contour length indicate that, as observed in solution measurements, AcP unfolds mechanically in an essentially two-state manner with no apparent unfolding intermediates. This conclusion is supported further by the results we obtained from the Go-model simulations (see below).

Consistent with its structural characteristics, AcP unfolds at forces which are significantly smaller (up to six folds) than those recorded for mechanically stable proteins or protein domains pulled at similar speeds. Compared to other α/β and all-β proteins that lack force-resistant topologies, AcP exhibits an average mechanical stability. An example for one such protein is the small ribonuclease barnase, which like AcP has an anti-parallel, five-stranded β-sheet core.

Pulled at 300 nm/s, barnase unfolds at a force of 70 pN (43), compared to ~50 pN which is required to unravel individual modules in $[AcP]_4$ when pulled at a similar speed (267 nm/s). An α/β protein that has a mechanical stability lower than AcP is barstar - barnase natural inhibitor. Compared to barnase and AcP, barstar possesses a smaller β-sheet comprising only three strands. Moreover, the strands directly exposed to the force are located at the edges of the sheet and, therefore, connected to it only on one side. As a result, barstar exhibits very poor mechanical stability, unfolding at forces close to or lower than the detection limit of the AFM (~10 pN) even when pulled at 400 nm/s (48), a rate at which AcP is found to yield to forces four-five times higher. Another relevant example is the first domain of synaptotagmin, C2A, which has a β-sandwich structure consisting of eight antiparallel strands. The terminal strands of this all-β domain are directly hydrogen bonded but the bonds are oriented in parallel rather than perpendicular to the direction of the force, allowing for sequential breakage ('unzipping') of the bonds [as opposed to shear topologies where bonds are loaded in parallel]. This mechanically feeble topology is partially compensated for by the fact that one of the force-bearing β-strands is stabilized at its outer side by interactions with a neighboring strand (48). Notably, one of the force-bearing strands of AcP, the N-terminal strand, is likewise flanked, at both sides, by neighboring strands to which it is connected by multiple hydrogen bonds (Fig. 1). As a result of this stabilizing effect, both C2A and AcP exhibit a reasonable (and similar) mechanical stability, unfolding at ~60 pN when pulled at 600 nm/s (ref (2) and Fig. 3 *C*), albeit lacking any other topological stabilizing motif in their force-bearing regions. To put this 'reasonable' mechanical stability in context, we note however that the mostly α-helical protein T4 lysozyme (19, 55), which derives its stability predominantly from inter-helical hydrophobic interactions, unfolds at forces very similar to those needed to unravel AcP.

The unfolding rate of AcP under force does not correlate with its unfolding rate in solution

The mechanical stability of proteins is a kinetic rather than a thermodynamic property. The relevant parameter (if any) for comparing results obtained from single-molecule pulling experiments to those obtained from bulk solution measurements is therefore their thermal unfolding rate. hmAcP unfolds in solution rather slowly, with a rate constant of $\sim 1 \cdot 10^{-4}$ s^{-1} (30, 32, 33). However, the spontaneous (zero force) unfolding rate extracted from the MC simulations for the forced unfolding of AcP (Fig. 3 *C*) was $3 \cdot 10^{-2}$ s^{-1}, more than two orders of magnitude higher. Faster unfolding rates under applied mechanical force were noted previously for other proteins, including barnase (43), ubiquitin (50), and protein L (45). Although the error associated with the estimate of unfolding at zero force could be significant, it is highly unlikely to account for the very large differences in unfolding rates observed for all of these proteins. One possibility is that there exists an outer energy barrier, which rate-limits the transition at zero force but is suppressed throughout the range of loading rates used in the pulling experiments. The experimentally accessible dynamics is thus dominated by inner barriers (54, 56, 57), giving rise to a faulty unloaded rate constant. However, given that AcP apparently unfolds mechanically in a two-state fashion, only a single prominent energy barrier needs to be crossed *en route* to the unfolded state, rendering this latter scenario unlikely. Another possibility is that the strict exponential decrease of unfolding time with applied force, which is assumed for pulling rates used in the AFM experiments [where unraveling occurs at time-scales much longer than those needed for diffusive relaxation], is invalid. Deviation from this Bell-Evans-type behavior is expected if the energy barrier for the transition is not sharp and, hence, not stationary with force. This may result in a more moderate dependence of the unfolding rate on the force at low

extension rates (58), giving rise to a slower rate of unfolding when extrapolated to zero force. Finally, the discrepancy may reflect genuine dissimilarities in unfolding pathways under the two sets of conditions.

The barrier for mechanical unfolding of AcP is located 0.6 nm away from the folded state

An important characteristic that can be derived from the force-velocity curves (also called force spectra), such as those shown in Fig. 3, *C* and *D*, is the distance between the folded and the transition state along the reaction coordinate set by the force (54, 56, 59). For AcP, the values we obtained for this parameter, denoted x_u, from the fit of the MC simulations to the experimental data and from the Go-model simulations (performed at relatively low pulling rates, see below) were 0.6 and 0.65 nm.

Recently, the correlation between x_u and protein secondary structure and topology was analyzed for a large set of proteins, using both experimental data and results obtained from pulling simulations employing off-lattice Go-like models (47). The analysis revealed that all β or α/β proteins have x_u values that range from 0.2 to 0.5 nm (barnase, for example, has an x_u value of 0.33 nm), whereas more compliant all α proteins have larger values, between 0.7 and 1.5 nm. The values we derived in this work for AcP fall in between those derived for the two groups. The aforementioned analysis also revealed that x_u scales linearly with the helix content of the protein. Using the linear regressions derived by Li et al. for the experimental ($R = 0.91$) or simulated ($R = 0.94$) data sets, the values we obtain for AcP are 0.39 and 0.37 nm, respectively, well below the value we derived from our data. We believe that the significant deviation of x_u of AcP from the expected dependence on helical content, which also accounts for the segregation of this protein from other α/β proteins, is due to the long loop (7 aa) that connects the short C-terminal β-strand (β_T) to strand 4, which is located at the other side of the β-sheet (Fig. 1 *C*). We propose that this long loop, which is poorly mechanically connected to other structural elements in the protein, substitutes helical structures in the sense that it offers a high compliance to the force and, thus, attenuates its loading onto strand 4 and, thus, to the β-sheet core of AcP. Indeed, if the amino acids within this loop and within β_T are considered to proxy α-helical regions, the corresponding x_u values generated by the aforementioned linear fits become very close to the 0.6-0.65 nm value we derived from the measurements and Go-model simulations. It thus appears that x_u is determined primarily by structural elements which are least resistant to mechanical deformation, such as α-helices and loop regions, or poorly connected strands, which likely yield to the applied force first. Next, the major resistors, namely significantly hydrogen-bonded β-strands and hairpin loops, submit, leading to unfolding of the protein. This is consistent with the fact that, as opposed to the excellent correlation found in the analysis described above between x_u and helical content, only poor correlations were found between the former and the β-content of proteins in the data set.

Ligand binding to AcP attenuates its forced unfolding to a similar extent as measured in bulk assays

Binding of inorganic phosphate is known to stabilize the native state of AcP. This stabilization results from an attenuated unfolding process; the rate of folding is unaffected by the presence of the anion (33).

Fig. 3 *D* shows the force spectrum obtained for poly-AcP in the presence of 10 mM phosphate, together with a spectrum obtained in the absence of the anion. As can be seen, the presence of phosphate increased the measured unfolding forces by a more or less constant value throughout the entire range of pulling speeds, resulting in a force spectrum that is shifted up relative to that obtained in the absence of the ligand. As the slope of the force spectrum relates to the position of the transition state along the direction of the force (see inset of Fig. 3 *C*), this means that the binding of phosphate does not affect this characteristic of the protein. This, in turn, suggests that the bound anion does not change the surface area exposure of the transition state as compared to that of the ligand-free protein. The latter notion is in agreement with results obtained from solution assays, which indicate no significant changes in denaturant folding/unfolding *m* values (which report on differences in hydrophobic surface accessible to the solvent between the end states and the transition state) in the presence of phosphate (33). The fit of the simulations to the data yields an apparent unfolding rate, which is five times lower than that derived in the absence of phosphate. This is very close to the 5.8 times decrease in unfolding rate measured in solution in the presence of 2 mM phosphate dissolved in the same buffer as that used in our studies (33). Thus, the binding of phosphate stabilizes the folded state of AcP relative to the transition state to the same extent whether it is isolated or oligomerized and regardless of the way unfolding is triggered, namely by denaturant or by mechanical force.

A model for forced unfolding of AcP

To gain insight into the sequence of the events associated with the mechanical unfolding of AcP we performed C^{α}-Go-type and all-atom steered MD simulations. As a model, we used the solution structure of horse muscle AcP (Fig. 1). This protein differs from the human ortholog we used in the experiments in seven amino acids.

Go model. Go-model simulations were performed in the over-damped limit (see Supporting Material). This allowed us to study unfolding even at relatively low pulling speeds, the lowest one being only 2.6 times higher than the maximal speed used in the experiments. Force-extension profiles obtained at different speeds revealed a single stable peak (Fig. 4 *A*), indicating that, in accordance with the experimental results, mechanical unfolding of AcP proceeds without intermediates. Plotting the unfolding forces obtained from simulations carried out at different pulling speeds yielded a force spectrum consisting of two linear regimes that cross over at $v_c \sim 3 \cdot 10^5$ nm/s (Fig. 4 *B*). The x_u value corresponding to the low-force regime of the spectrum is 0.65 nm, in good agreement with the experimentally determined value of 0.6 nm. As only one peak is observed in the simulated force-distance traces, the presence of the second (high-force) regime in the force spectrum is surprising and is likely to be artificial. This is probably due to the high loading rates associated with this regime, which may alter the relaxation dynamics of the protein and, therefore, the characteristic dependence of the force on extension speed (see Eq. 3 in the Supporting Material).

Figures 4 *C* and 4 *D* show the dependence of the fraction of native contacts (NC) present in secondary structures of AcP, as well as between nine pairs of these structures, as a function of extension of the protein. As can be seen, unfolding commences by unraveling of the C- terminal β-strand, β_T, which quickly loses its native contacts upon extension. This is swiftly followed by the simultaneous and likewise cooperative unfolding of strands β_1, β_2, and β_4. The remaining

strand of the sheet, β_3, survives longer and exhibits a biphasic transition, reflecting loss of contacts with β_1 and β_2. The two helices present in AcP unfold last, in a stepwise manner.

All-atom model. Four trajectories were generated in these simulations, which were carried out at a pulling speed of 10^9 nm/s. Here, the process of unfolding was followed by monitoring the number of hydrogen bonds present in secondary structures. The force-extension profiles obtained in the four runs revealed three peaks, the first of which is located at $\Delta R \approx 2.5$ nm, not far from the position of the peak observed in the profiles generated by the Go-model simulations ($\Delta R \approx$ 1.8 nm). The other two peaks, which are not observed experimentally or in the traces derived from the Go-model simulations, are likely to be artifacts produced by the high extension speed employed in the all-atom simulations. As can be seen in Figures 5 and 6, unfolding is initiated at β_T, following breakage of its hydrogen bonds with β_2. The rest of the protein then unfolds in the following manner: $\beta_4 \rightarrow (\beta_1, \alpha_2) \rightarrow (\beta_2, \beta_3, \alpha_1)$.

While the unfolding pathways predicted by the two methods are not identical, possibly reflecting differences in pulling speeds (60) or models (61), both indicate that unfolding of AcP is initiated at β_T, that β_4 unfolds soon afterwards, and that β_3 (and, possibly, β_2) and the two helices persist till the late stages of unfolding. That β_T is the first element of AcP that yields to the force is not surprising since, as mentioned before, this short, force-bearing strand is positioned at the edge of the β-sheet and is connected to it through few hydrogen bonds with β_2 (Figs.1 *A*). Located in this strand is Phe94 - one of few residues that determine the folding transition state architecture of AcP by promoting the establishment of a native-like interaction network (33, 36, 37). Unraveling of this strand is thus expected to perturb this network, facilitating disruption of the major hydrophobic core of AcP. The poor ability of β_4 to withstand force is likewise expected as, like β_T, it is also located at the edge of the sheet and, therefore, forms hydrogen bonds with only one strand (β_1). It is also connected, through a loop, to β_T and, thus, is subjected directly to the force once β_T unravels. Unfolding of β_4, in turn, should destabilize the other force-bearing unit of AcP, β_1, with which it interacts through hydrogen bonds. As discussed above, the transmission of the force from β_T to β_4 is likely to be damped by the long loop that connects them, leading to an unexpectedly large x_u value. In contrast to β_T and β_4, β_3 lies at the center of the sheet and is hydrogen bonded to both strands 1 and 2. The mechanical stability of β_3 is likely increased further by the hairpin loop that connects it to β_2 and by the two contacts it makes with Lys57 and Val58 in α_2, which, as mentioned above, are maintained through most of the unfolding process (Fig. 4 *D*). These contacts also contribute to the preservation of α_2 till the very late stages of unfolding. We believe, however, that the strong persistence of this helix, as well as of α_1, is mostly a manifestation of the fact that the two helices are topologically segregated from the rest of the protein and are, therefore, relatively autonomous. This interpretation is consistent with results obtained from MD simulations, which showed that formation of α_1 and α_2 during folding of AcP is distinct from the process of nucleation (37), even though their stabilization can affect both folding and unfolding rates (34, 39).

As for the effect produced by phosphate binding on the unfolding rate of AcP, we suggest that it is due to stabilization of the N- and C-terminal strands of AcP. In all AcPs, the phosphate group of the substrate binds to a conserved arginine residue (Arg23 in hmAcP), which makes contacts with Val96, in β_T of hmAcP (37), with Thr100 and Tyr101, in β_T of AcP of the archaeon

Sulfolobus solfataricus (26), and with different residues located in β_T (or its equivalent regions) of all other AcPs whose structure is available (our own observation, following analysis using CSU (62)). This residue is located at the N-terminal end of helix 1, adjacent to the C-terminal end of the catalytic loop (Fig. 1 *C*) which, in turn, is connected to the N-terminal strand β_1. Analyses of bovine (common-type) (22) and archeal (27) AcPs, crystallized in the presence of sulphate or formate, revealed that the anion forms a salt bridge with the guanidinium group of Arg23, as well as hydrogen bonds to backbone amides of this and several other residues in the catalytic loop. In addition, three structured water molecules, which likely accompany the anion, form hydrogen bonds with the anion and with backbone amide groups in several residues in the catalytic loop. They also form hydrogen bonds with the side-chain of a conserved asparagine residue (Asn41 in hmAcP, Fig. 1 *C*), located at the C-terminal of the second β-strand, which plays an essential role in catalysis (as it orients the catalytic water molecule that serves as the attacking nucleophile for hydrolysis of the carboxyl-phosphate bond (22)). The result is an extensive interaction network that strongly stabilizes Arg23 and the catalytic loop. We propose that this stabilization renders β_1 and β_T, which are the force-bearing units of AcP, more resistant to force. Based on the good correspondence in the extent of deceleration of unfolding by phosphate binding observed in pulling and in chemical denaturation experiments, we further suggest that this stabilization also underlies the slower unfolding of phosphate-bound AcP in the absence of external force.

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FIGURE LEGENDS

Figure 1 Structure of AcP. (*A*) Solution structure of horse muscle AcP (PDB code: 1APS (21)). Secondary structures (defined following DSSP) are represented as ribbons and backbone hydrogen bonds in the β-sheet are shown as dashed lines. The force-bearing units are the N- and C-terminal strands. (*B*) Topology diagram. AcP adopts a rather uncommon α/β sandwich fold elaborated by two intercalating $\beta\alpha\beta$ units forming an antiparallel β-sheet with a 4-1-3-2-5(β_T) strand topology. (*C*) Structural determinants for forced unfolding of AcP. The long loops that follow and precede the N- and C-terminal, force-bearing β-strands are colored in cyan and red. The former, referred to as the catalytic loop, adopts a cradle-like conformation and constitutes the active site of the enzyme. Also shown are the conserved Arg23 (orange) and Asn41 (yellow) residues, which flank the cradle and function in binding the substrate phosphate group and the catalytic water molecule, respectively.

Figure 2 Properties of poly-AcP. Individual modules in the polymeric construct preserve the structure and catalytic activity of the innate, isolated protein at room temperature (25 ºC), as demonstrated by comparing their far-UV CD spectrum (*A*, dashed line) and ability to hydrolyze the AcP substrate benzoyl phosphate (*B*, open circles) to those of monomeric AcP (solid line and solid circles, respectively).

Figure 3 Forced unfolding of poly-AcP. (*A*) A typical force-extension curve obtained by stretching individual AcP polymers at 100 nm/s. The high force peak seen at the beginning of the extension profile reflects non-specific interactions between the AFM tip and the mounting surface. The solid lines superimposed on the rising parts of the peaks are fits to a worm-like-chain model. (*A, inset*) Contour length increments upon domain unraveling obtained from the fitting (v_c = 267 nm/s) (*B*) Frequency histograms of unfolding forces recorded at different pulling speeds. (*C*) Dependence of the most probable force for unfolding, taken as the maximum of the unfolding force distributions, on the pulling speed. Albeit not seen in all data points shown, error bars are included. The best fit to the data from the Monte Carlo simulations (solid lines in the main figure and inset) was obtained using k_u^0 = 0.03 s^{-1} (main figure) and x_u = 0.6 nm (inset). It was shown that very high pulling speeds could be associated with distance-dependent drag forces, which may lead to underestimation of the unfolding force at such speeds (63, 64). Analysis we performed reveals that the deviation expected, even for the highest pulling speed used in the experiments described in this work, lies within the thermal noise error. (*D*) Force spectra obtained for poly-AcP in the absence (solid rectangles, solid line) or presence (open circles, dashed line) of 10 mM P_i. The presence of the ligand stabilizes the native structure of the protein, leading to deceleration of the unfolding reaction. However, the position of the transition state ensemble along the force-set unfolding pathways is not affected by the ligand, as evident by the fact that the slope of the force spectrum is unaltered.

Figure 4 Summary of the results obtained from the Go-model simulations. (*A*) Representative force-extension profiles. The presence of a single, stable peak in the profiles, implies a two-state unfolding process. As expected and as observed experimentally, the height of the peak increases with pulling speed. (*B*) Force spectrum derived from simulations conducted at pulling speeds ranging between $2.6 \cdot 10^4$ and $7.3 \cdot 10^6$ nm/s. As discussed in the text, the linear regime corresponding to pulling speeds greater than ~$3 \cdot 10^5$ nm/s is likely to be an artifact due to the fast loading of the force onto the protein in this regime. (*C* and *D*) Dependence of native contacts

(NC) present in secondary structures of AcP (*C*) and between nine pair combinations of them (*D*) on extension (v_c = 2.6·10^4 nm/s). The arrow denotes the position of the peak observed in the force-extension traces.

Figure 5 Breakage of hydrogen bonds between β_1 and β_2 at the commencement of unfolding, as revealed by snapshots taken from trajectories obtained from four runs of steered MD simulations using an all-atom model of AcP (v_c = 10^9 nm/s). The upper and lower panels correspond to structures obtained just before and after the first peak in the corresponding force-extension profiles.

Figure 6 Fraction of hydrogen bonds in secondary structures of AcP as a function of extension, derived from the four runs of the all-atom MD simulations.

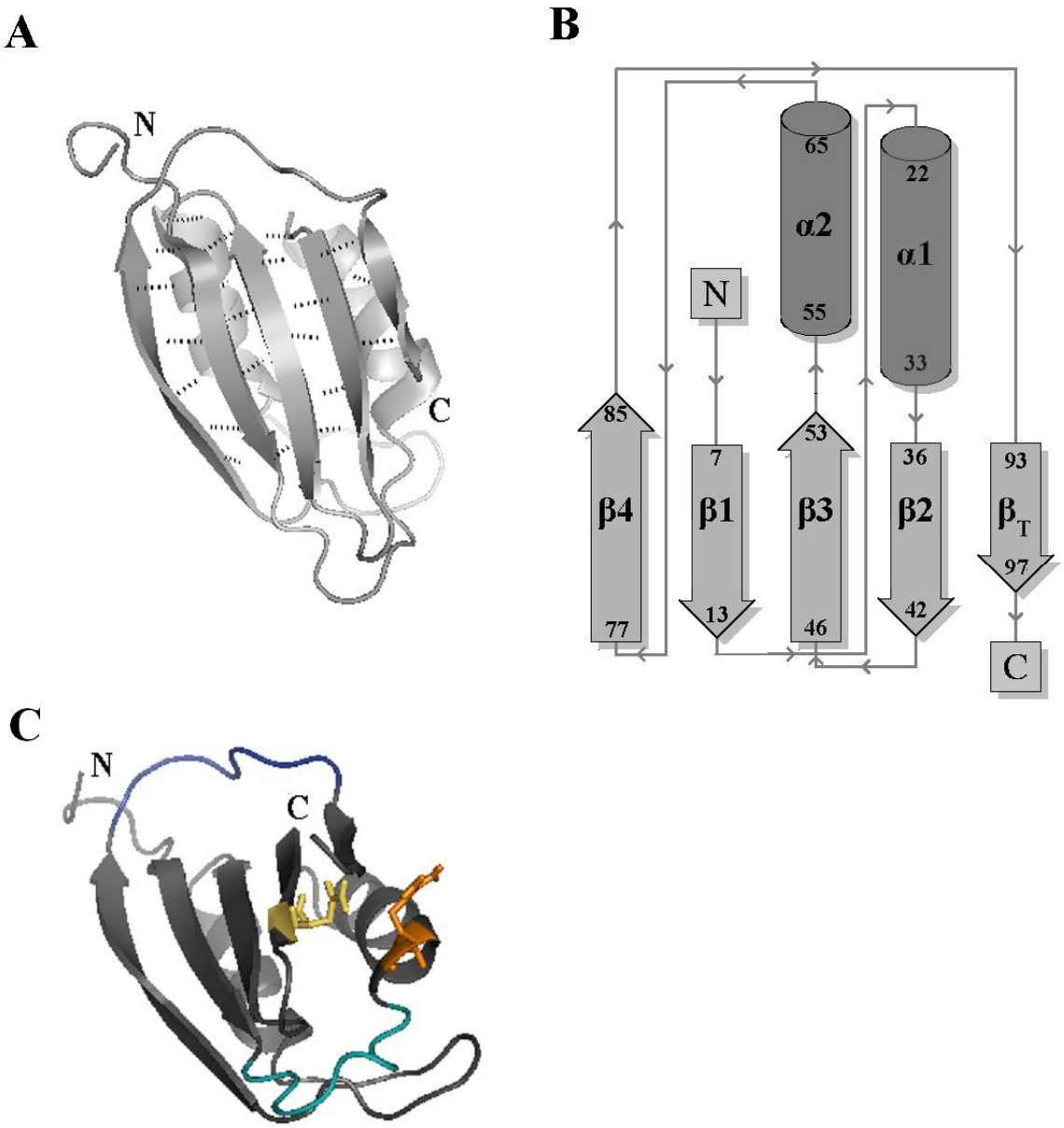
A
N
C
B
65
α2
55
22
α1
33
N
85
β4
77
7
β1
13
53
β3
46
36
β2
42
93
β_T
97
C
C
N
C

Fig. 1

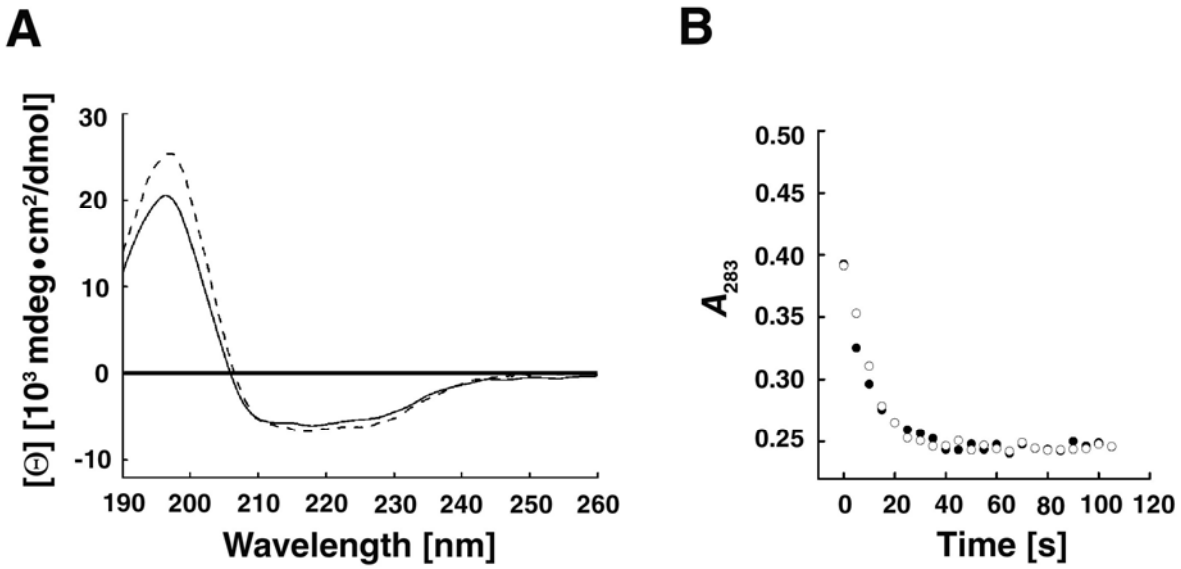
A
B
[Θ] [10³ mdeg • cm²/dmol]
30
20
10
0
-10
190
200
210
220
230
240
250
260
Wavelength [nm]
A283
0.50
0.45
0.40
0.35
0.30
0.25
0
20
40
60
80
100
120
Time [s]

Fig. 2

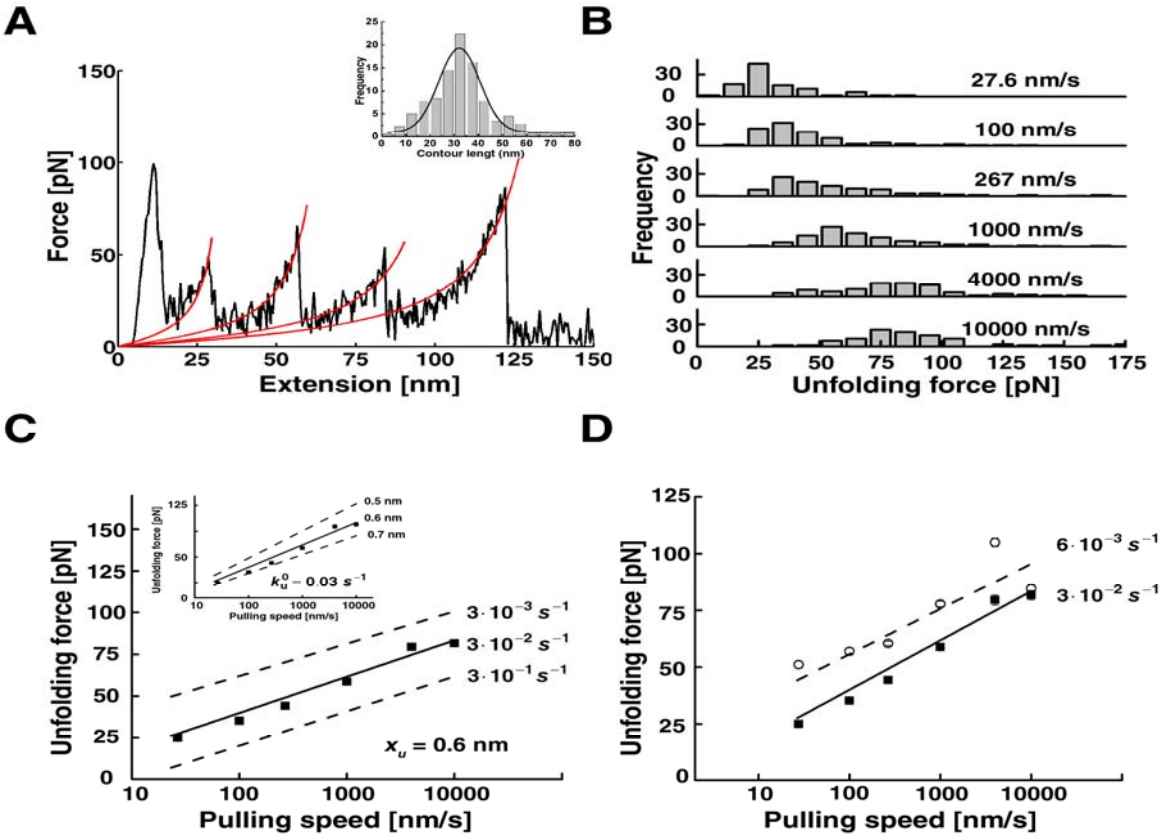
A
Force [pN]
Extension [nm]
Frequency
Contour lengt (nm)
B
Frequency
27.6 nm/s
100 nm/s
267 nm/s
1000 nm/s
4000 nm/s
10000 nm/s
Unfolding force [pN]
C
Unfolding force [pN]
Pulling speed [nm/s]
0.5 nm
0.6 nm
0.7 nm
$k_u^0 = 0.03\ s^{-1}$
$3 \cdot 10^{-3}\ s^{-1}$
$3 \cdot 10^{-2}\ s^{-1}$
$3 \cdot 10^{-1}\ s^{-1}$
$x_u = 0.6$ nm
D
Unfolding force [pN]
Pulling speed [nm/s]
$6 \cdot 10^{-3}\ s^{-1}$
$3 \cdot 10^{-2}\ s^{-1}$

Fig. 3

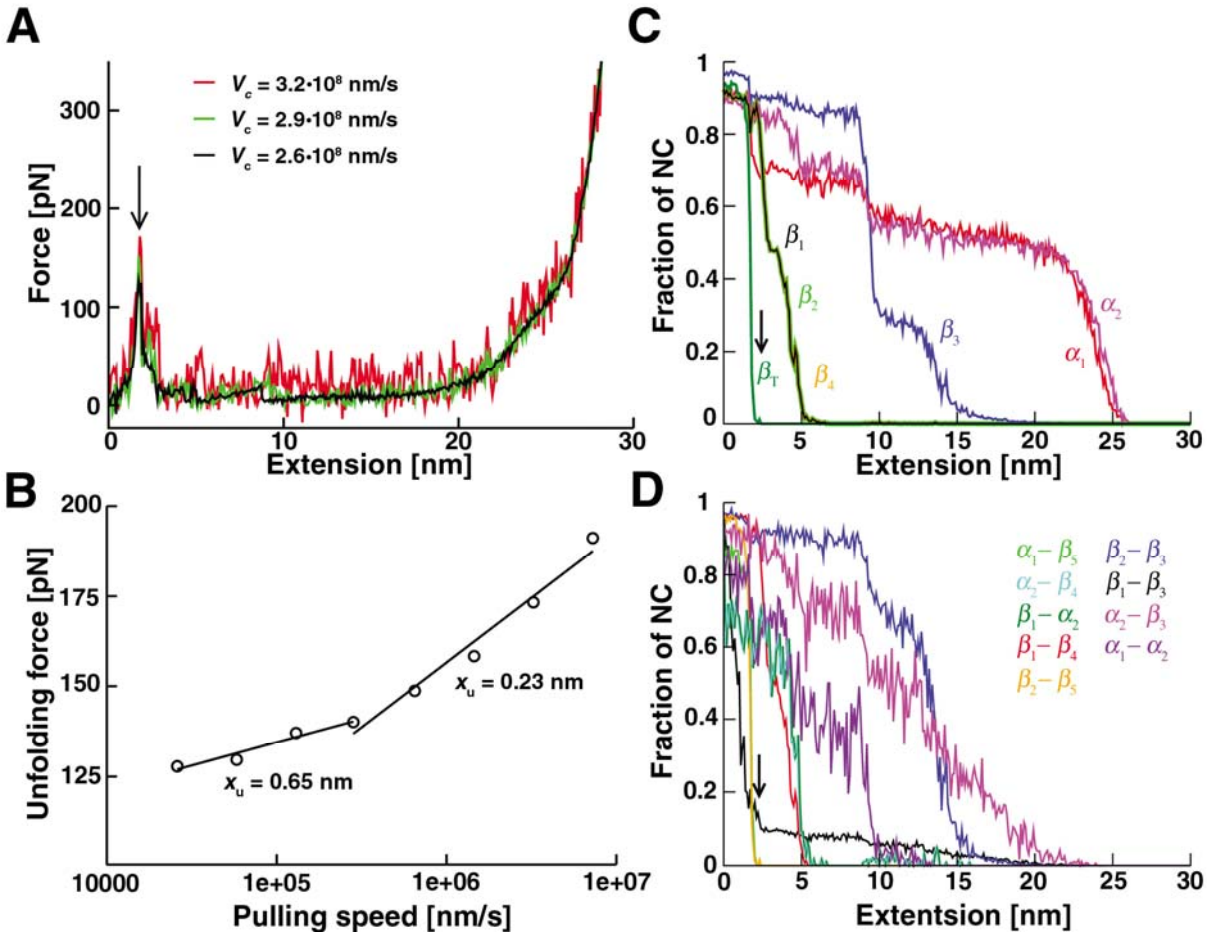
A
V_c = 3.2·10⁸ nm/s
V_c = 2.9·10⁸ nm/s
V_c = 2.6·10⁸ nm/s
Force [pN]
Extension [nm]
B
Unfolding force [pN]
x_u = 0.65 nm
x_u = 0.23 nm
Pulling speed [nm/s]
C
Fraction of NC
β_1
β_2
β_3
β_4
β_T
α_1
α_2
Extension [nm]
D
Fraction of NC
$\alpha_1-\beta_5$
$\alpha_2-\beta_4$
$\beta_1-\alpha_2$
$\beta_1-\beta_4$
$\beta_2-\beta_5$
$\beta_2-\beta_3$
$\beta_1-\beta_3$
$\alpha_2-\beta_3$
$\alpha_1-\alpha_2$
Extentsion [nm]

Fig. 4

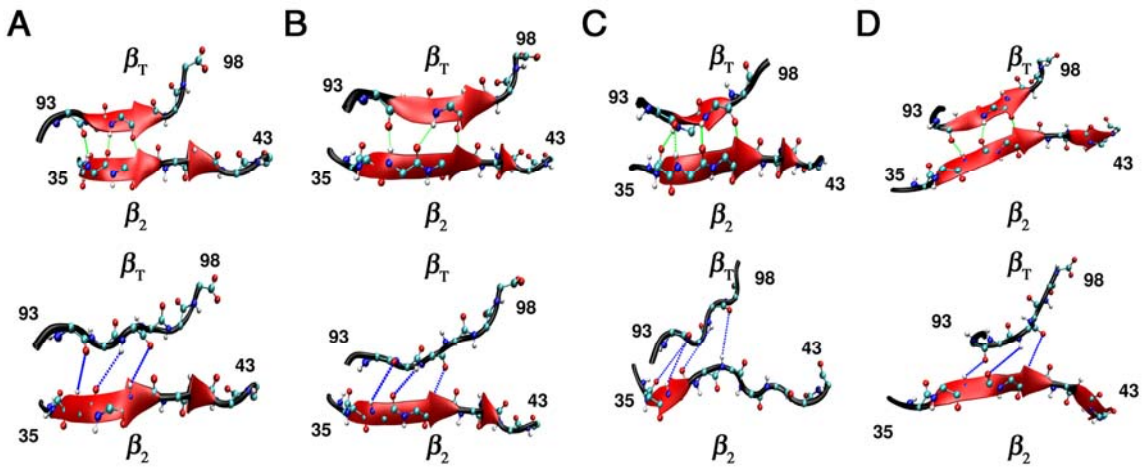

Fig. 5

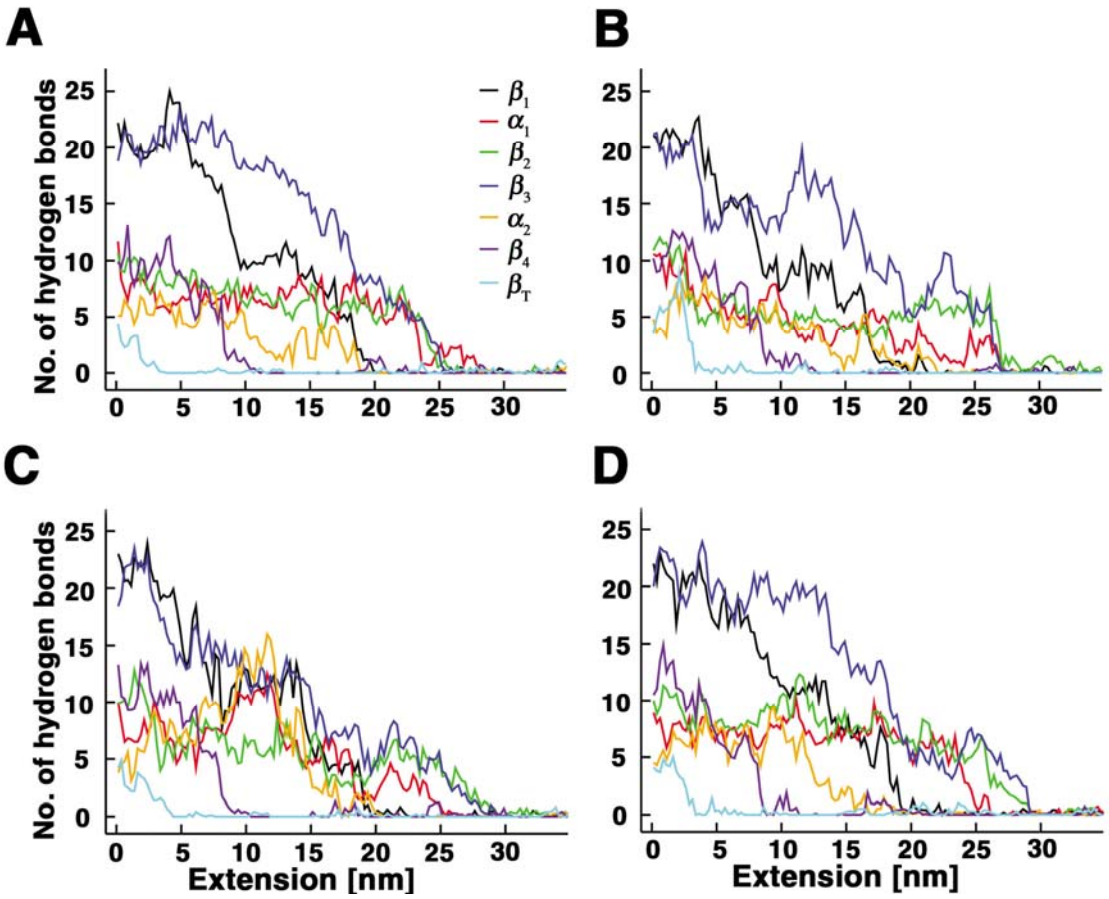
A
B
C
D
No. of hydrogen bonds
Extension [nm]
β_1
α_1
β_2
β_3
α_2
β_4
β_T
0
5
10
15
20
25
30

Fig. 6