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How flexible is α-actinin's rod domain?

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Abstract: α−actinin, an actin binding protein, plays a key role in cell migration, cross-links actin filaments in the Z-disk, and is a major component of contractile muscle apparatus. The flexibility of the molecule is critical to its function. The flexibility of various regions of the molecule, including the linker connecting central subunits is studied using constant force steered molecular dynamics simulations. The linker, whose structure has been a subject of debate, is predicted to be semi-flexible. The flexibility of the linker is compared to all possible segments of equal length throughout the molecule. The stretching profile of the molecule at different forces suggests that loops and regions adjacent to the loops are much more rigid than the helices in the protein. Amino acid composition analysis of most flexible and most rigid regions of the molecule reveals that the rigid regions are rich in Ser, Val and Ile whereas the flexible regions are rich in Ala, Leu and Glu.

1 Introduction

Actin filaments play a central role in cell migration. The assembly and disassembly of the actin network at the leading edge of the cell forms the basis of cell motility. In muscle cells, actin filaments are a major component of the contractile apparatus of skeletal muscle [Borisy and Svitkina (2000), Geeves and Holmes (1999)]. The functioning of this actin network depends upon the structure and function of actin binding proteins. One such protein, α-actinin is a ubiquitously expressed protein, that binds and cross-links actin filaments in both muscle and non-muscle cells [Otto (1994)]. In cardiac and skeletal muscles, α-actinin is present in the Z-disk, where it serves to cross-link the anti-parallel actin filaments [Masaki, Endo, and Ebashi (1967)]. On the other hand, in non-muscle cells, α-actinin organizes the cortical cytoskeleton adjacent to membrane-associated structures such as zonula adherens and tight junctions. α-actinin also interacts with various other cytoskeletal and membrane bound proteins, and is believed to be involved in linking actin cytoskeleton to the membrane [Otey, Pavalko, and Burridge (1990), Carpen (1992), Heiska (1996), Wyszynski (1997)]. α-actinin has also been found at the at cell adhesion sites, focal contacts and the leading edge in migrating cells [Knight (2000)].

α−actinin belongs to a family of CH (calponin-homology) domain proteins that have a characteristic actin binding domain. Structurally, α-actinin consists of an amino terminus (which contains two CH domains), a central rod like domain, and a calmodulin type C-terminal domain [Castresana and Saraste (1995)]. The C- and the N-termini domains form the actin binding head of the molecule, which are connected together by spectrin-like domains of the central region. This rigid connection between the head domains is often referred to as a "rod" domain, due to its elongated structure and rigidity.

The rod domain in α-actinin is critical for its function, as it determines the distance between the cross-linked subunits of the actin network. It has also been reported to provide interaction sites for trans-membrane receptors [Otey, Pavalko, and Burridge (1990), Carpen (1992), Heiska (1996)], [Pavalko (1995), Pavalko and LaRoche (1993), Papa (1999), Galliano (2000)]. The three dimensional structure of the central repeats in the rod domain has recently been solved by Saraste and co-workers [Djinovic-Carugo (1999)]. The 2.5 Å structure suggests the presence of a helical linker that links the two central repeats to form a symmetric and anti-parallel dimer. The structure and flexibility of this linker region is critical for the overall flexibility of the molecule. There has been some debate regarding

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the structure of the linker, shown to be helical in both α-actinin rod and α-spectrin [Djinovic-Carugo (1999), Grum (1999)]. Initially, the linker was suggested to be non-helical [Speicher and Marchesi (1984)], however recent studies have suggested lack of any discontinuity in the helix that connects the repeats joined by the linker [Djinovic-Carugo (1999), Grum (1999)]. The flexibility of this linker region has been the building block of the models used to explain the overall flexibility of the molecule, and hence the function of spectrin repeats in spectrin and α-actinin is associated with the structure and the flexibility of the linker [Grum (1999)].

Though there is clear evidence for the helicity of the linker in α-actinin rod domain, the flexibility of this linker still remains unclear. Is this helical linker a rigid helix? Or is the helical linker a weak helix, such that it will undergo a conformational change to a random coil under small mechanical stresses? How does the flexibility of this helical region compare with other helical or coil regions of the molecule? Are the helices more or less flexible than the coil regions of the molecule? These questions are fundamental to our understanding of the flexibility of the rod domain, and have not been answered as yet. In addition, a deeper understanding of the rigidity of the rod domain requires that we address the issue of other flexible and rigid regions of the molecule.

Using constant force steered molecular dynamics, this paper addresses the issue of the flexibility various segments of the molecule, including that of the linker region. In recent years, constant force and constant velocity steered molecular dynamics have provided a detailed picture of protein behavior under mechanical stress [Krammer (1999), Lu (1998), Lu and Schulten (1999), Lu and Schulten (2000) Lu (2000), Paci and Karplus (2000), Fowler (2002)]. These studies have given us an insight into the molecular level events, such as intermediate structure formation and preferred pathways adopted by the molecule as it is mechanically unfolded. Proteins such as spectrin, fibronectin and titin have been studied successfully with steered molecular dynamics and these computational studies have shown good agreement with AFM and optical tweezer experimental studies [Rief (1997), Rief (1998), Rief (1999), Rief, Gautel, and Gaub (2000), Altmann (2002), Smith, Finzi, and Bustamante (1992), Binnig, Quate, and Gerber (1986), Ashkin, Dziedzic, and Yamane (1987)].

In this study, we aim to understand the flexibility of various regions of α-actinin rod domain, including the flexibility of the helical linker, and we propose an explanation of our findings of flexibility of individual regions in terms of secondary structure and amino acid composition. We hope that this information will lead to better models of α-actinin's flexibility and will result in experimental studies that will test our hypothesis.

2 Methods

Simulation Methods:

Constant force steered molecular dynamics (SMD) simulations were performed on the two central repeats in the rod domain of the actinin monomer using CHARMM program employing PARAM 19 force field [Brooks (1983)]. The implicit solvent model used has been developed by Lazaridis and Karplus [Lazaridis and Karplus (1999)]. The pdb structure of the monomer (pdb ID : 1QUU) was minimized for 1000 steps, its temperature raised to 310 K for 100 ps and was equilibrated for another 100 ps. SMD simulations were carried out with the N-term as fixed and the alpha carbon of C-term pulled with a constant force ranging from 5 pN to 150 pN away from the N-term (in the direction of the C-terminus). The simulations at each force were carried out for $\sim$ 1ns, unless complete unfolding of the molecule was achieved before that. The coordinates were binned at 0.1ps and the results were analyzed using VMD version 1.8.2 [Humphrey, Dalke, and Schulten (1996)]. The simulation was carried out on Intel PIV cluster.

Sliding Window Analysis of Hexamer Segments : Since one of the goals of this paper is to address the issue of the relative flexibility of the linker region which is six residue in length, we use all possible continuous hexamers in the protein to study the flexible and rigid regions. In other words, the end-to-end distance of the linker under forces ranging from 5-150 pN was compared to the end-to-end distance of hexamer between residue 1-6, 2-7, 3-8, 4-9 etc as the entire protein is stretched. There were 242 hexamers in all, as the rod domain is 248 residues in length. The percent extension of all the residues under various mechanical stresses is shown in figure 5(a-k).

Scoring the Segments: Based upon percent extension, the segments were ranked from most flexible (highest percent extension under mechanical stress) to least flexible. Percent extension is computed by calculating the average extension of each hexamer in the last 100 ps of the

Table 1 : The top 15 most flexible and least flexible regions of the protein. The secondary structure assignment is based on DSSP algorithm [Kabsch and Sander (1983)]

Most Flexible		Least Flexible	
Segment Residues	Secondary Structure	Segment Residues	Secondary Structure
21-26	Helix	152-157	Coil/Loop
23-29	Helix	217-222	Coil/Helix
54-59	Helix	173-178	Helix
140-145	Helix	157-162	Coil/Loop
22-27	Helix	206-211	Loop
123-128	Helix	197-202	Coil/Helix
58-63	Helix	192-197	Helix next to Coil/ Coil
139-144	Helix	212-217	Coil/Loop
93-98	Helix	156-161	Coil/Loop
126-131	Helix	205-210	Loop
118-123	Helix	155-160	Coil/Loop
55-60	Helix	201-206	Coil/Loop
72-77	Helix	200-205	Coil/Loop
53-58	Helix	154-159	Coil/Loop
52-57	Helix	153-158	Coil/Loop

forced stretching of the protein and comparing it with the end-to-end distance of the hexamer in the native state. At each force, a segment that had greatest percent extension was given a score 1 and the segment that had the least extension was given the score 242. This analysis was carried out for all the forces under which the simulation was carried out, and an average score was computed from each hexamer. The segment with the lowest average score was considered "most flexible" while the segment with the highest average score was considered "least flexible". The top 15 most flexible segments and top 15 most rigid (least flexible) segments are listed in Table I. (The ranking of all the segments is listed in supplementary Table S-I). The first 15 and the last 15 segments were not considered in the analysis due to "end effects".

Residue Composition Analysis: The amino acid composition of the most and least flexible segments was analyzed by comparing the percent occurrence of a given residue in the top 15 most flexible and top 15 most rigid sequences.

3 Results and Discussion:

The percent longitudinal extensions of all the possible segments in the α-actinin rod domain at different external forces are compared to identify the regions of highest flexibility and regions which are most resistant to external stress. These extensions are also compared to the linker region of the protein. The segments are ranked in order of decreasing flexibility (supplemental Table S-I). The analysis shows that the segment between residues 21-26 is most flexible and segment between residues 152-157 is least flexible (Table I). The extension-time profile at various forces of these segments along with that of the helical linker and the entire protein is shown in Figure 1.

The Figure shows that whereas the helix and the helical linker completely stretch at 80-100 pN the "least flexible" region shows very little extension at that force. In addition, shorter time scales produce a lot more extension in the most flexible region, similar extensions are produced at much longer time scales in the rigid segment.

Flexibility of the linker region: The helical linker scores fairly high (rank = 16/242) among the hexamer segments. This suggests that the helical linker is fairly flexible, however it is not a completely flexible Gaussian random chain, or a chain that disorders at minimal stress, as has been modeled previously. In other words, our results argue that the flexibility of the helical region is compara-

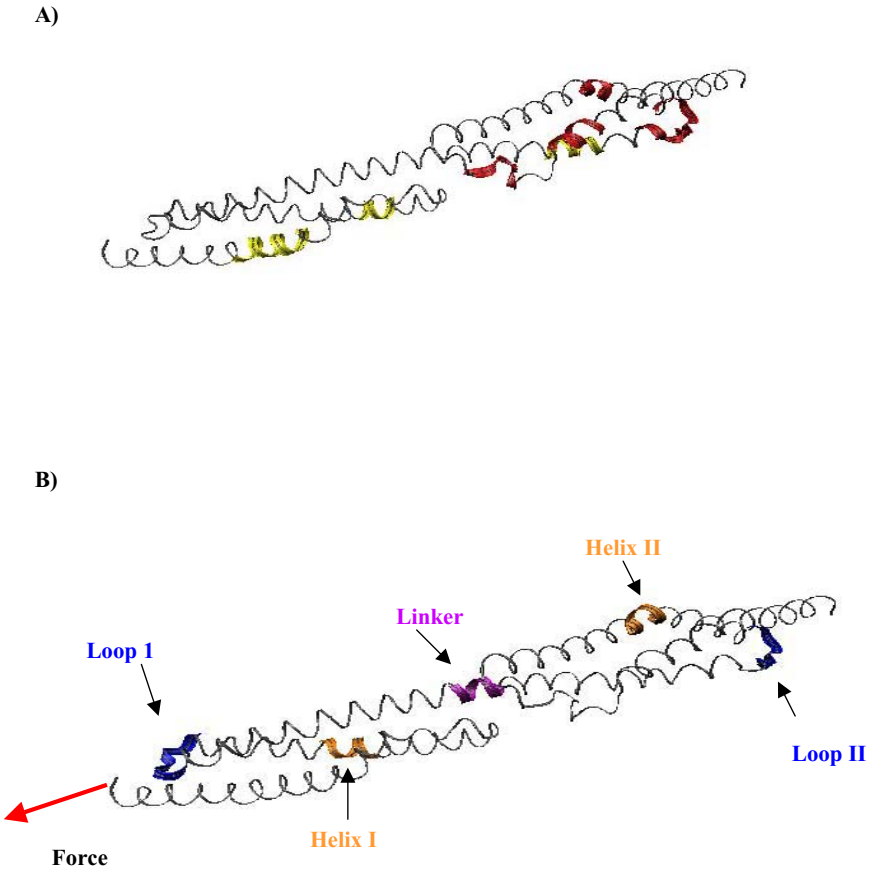


Figure 1 : **The extension of loops, helices and linker region under external stress:** The linker and the most flexible region show complete extension (unfolding) at 80-100 pN. For the rigid hexamer in the protein only forces greater than or equal to 150 pN generate an unfolded structure, and lower forces produce only slight extension. The protein as a whole also unfolds at ∼150 pN. The stretching of the helical linker, the flexible hexamer and the rigid hexamer are calculated as the whole protein is stretched (i.e. their stretching profile does not represent the stretching of isolated hexamer segments.)

ble to the most flexible regions of the protein, but it can not be modeled as an infinitely flexible linker connecting semi-flexible helices. The force-extension profile of the linker region (Figure 1) also shows that the overall profile of extension is very similar to the flexible helical region, and like the flexible helical region's profile, it is quite different from the force-extension profile of the rigid coil region.

Helices vs. Loops: A closer look at the most flexible and the least flexible regions of the protein shows another interesting feature of this protein. Whereas the most flexible regions are predominantly located in the helical regions of the protein, the least flexible regions are located either in the loop regions, or in the semi-helical/ coil regions adjacent to the loops (Table I. Fig 2).

In other words, our results show that in α-actinin rod domain the regions that are least flexible and are resistant to external stress are found in loops and coils, whereas the most flexible segments are found in the helical regions. This could also be a function of the direction of the application of force, as the loop regions are oriented essentially perpendicular to the direction of the applied force. We hope that further experiments and computational studies will establish as to whether this is a general phenomenon or only true in certain proteins.

Amino Acid Composition of the flexible and rigid regions : Our results tabulated in Table I show that a few hexamers which are least flexible, are located in the helical

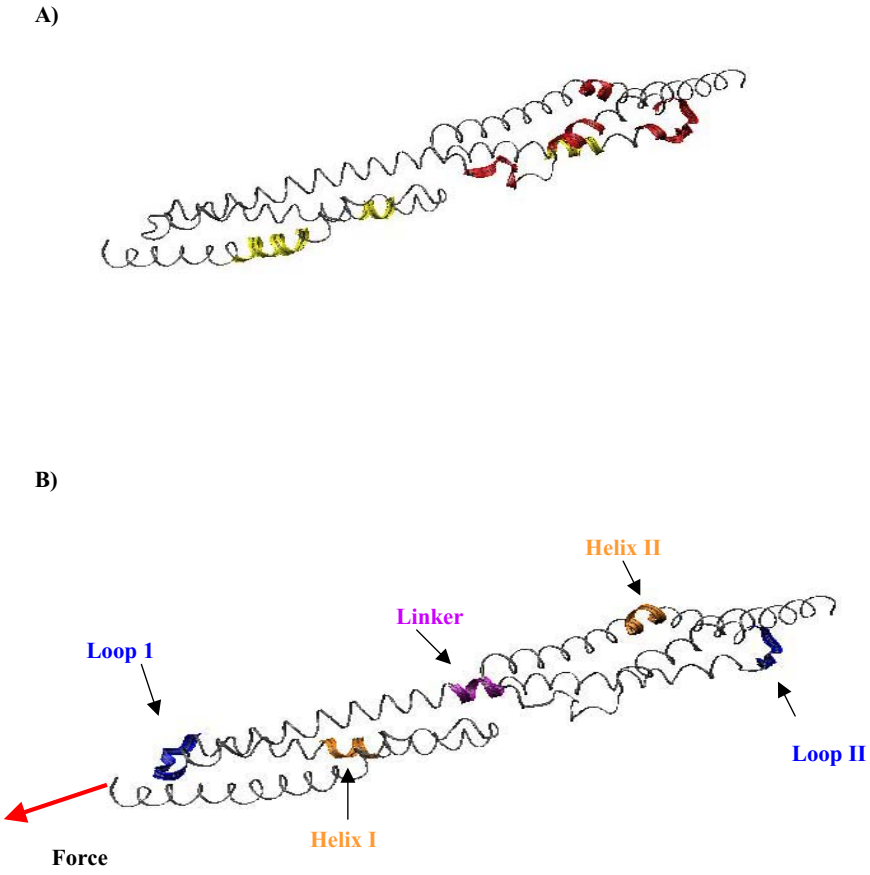


Figure 2 : **The flexible and rigid regions of a-actinin.**
(A) Color coded regions of highest and lowest flexibility. The red areas represent the four most rigid regions in the protein while the yellow colored segments represent the four most flexible regions of the protein. As mentioned in the methods section, the simulations were carried out on the monomer of α-actinin. (PDB code : 1QUU).
(B) The sample helical and loop regions are color coded. The helical region I is between amino acids 63-68, helical region II is between residues 177-182, the loop I is between amino acids 83-88 and the loop II is formed by amino acids 156-161. The linker comprises of amino acids 120-125. The direction of the constant external force is shown by the arrow. The helical regions are shown in orange, the two loops in blue and the linker is shown in purple.

regions. In other words, though overall the loop regions are more rigid than the helices, some helical hexamers are also among the 15 least flexible regions of the protein. We analyze the amino acid composition of the 15 most flexible and 15 most rigid hexamers in the protein. The results are summarized in Figure 3.

The results show that the most commonly occurring amino acids in the flexible regions and the rigid regions are in fact quite different. The 15 most rigid hexamers, which represent different loop regions or regions adjacent to loops are rich in Ile, Ser and Val, residues which are either absent or occur with relatively lower frequency ($< 20\%$) in the flexible regions of the protein. On the other hand, regions of highest flexibility are rich in Ala, Glu and Leu, amino acids which have less than 20% occurrence frequency in the rigid regions. These results are quite interesting as both the 15 most rigid and flexible segments are from different parts of the protein, yet they both contain a higher propensity of certain amino acids which are absent in their counterparts. In other words, our analysis suggests that the flexibility and rigidity of various regions in the α-actinin rod domain does not only have to do with the secondary structure, but also with the amino acid composition of these regions. We hope that further computational and experimental studies will pro-

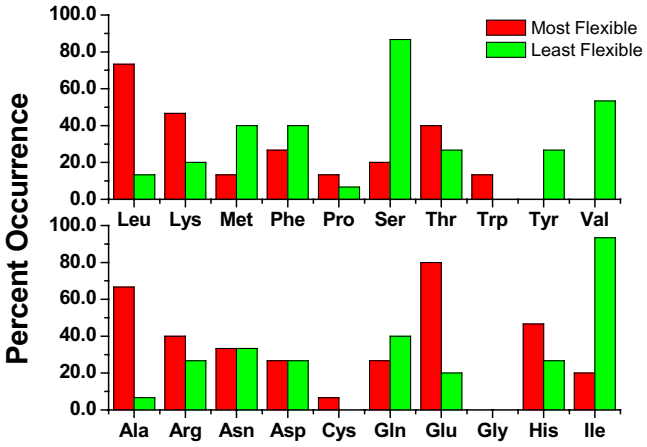


Figure 3 : **Amino Acid Composition of Rigid and Flexible Regions:** The amino acid composition of the top 15 most flexible regions and the top 15 least flexible regions is shown in a bar chart. The most commonly occurring residues in the most flexible regions, namely Ala, Leu and Glu are either completely absent or are rarely present in the rigid regions. Similarly, Ile, Ser and Val occur frequently in the rigid regions and are either absent or rarely present in the flexible regions of the protein.

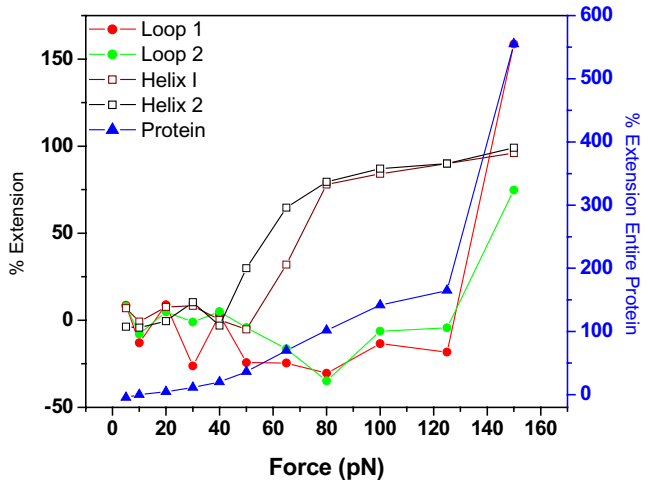


Figure 4 : **Percent extension vs. Force for loops and helices.** The percent extension of the loops shows a very similar profile to that of the entire protein, whereas the two helices (which only represent a sample, other helical regions show a similar trend) unfold at a much lower magnitude of force, suggesting that the loops are more rigid than the helices and perhaps are the limiting factor in the overall protein unfolding.

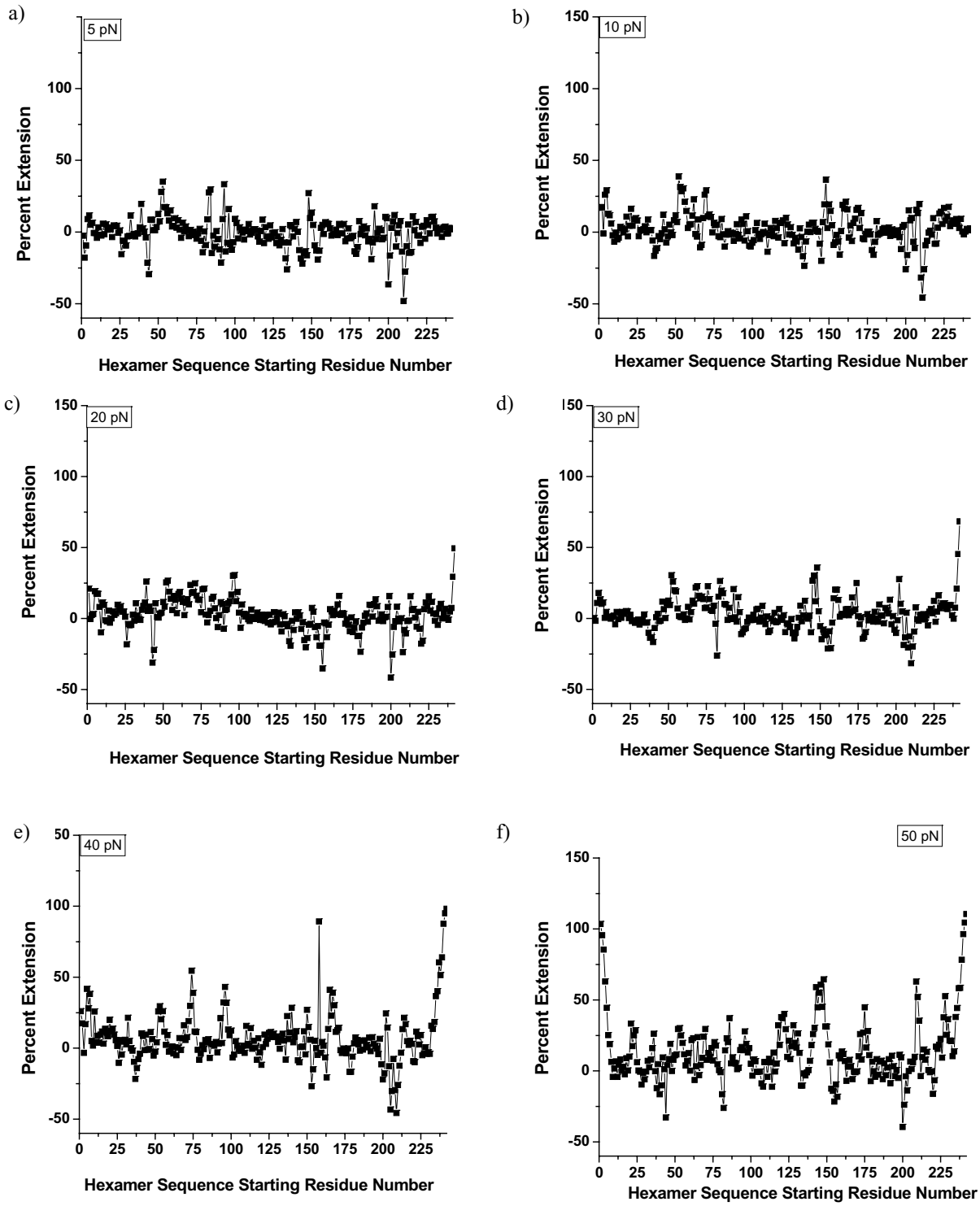
vide insights into the possible molecular-level reasoning for this observation.

Force required for stretching of Loops and Protein Unfolding : Another interesting observation of our simulation is the similarity in magnitude of force required to unfold the loops and the magnitude of the force required to unfold the protein. The percent extension of two sample loops and two sample helices (within the protein) is compared to the percent end-to-end extension of the entire protein (Figure 4).

It is interesting to note that whereas these sample helical segments (the results for other segments are similar, data not shown) show complete stretching at 80-100 pN the loops do not show complete extension until 150 pN, which is the magnitude of the force required to unfold the entire protein molecule. This feature is quite interesting and suggests that perhaps for proteins with loops and helices, the force required to stretch the loops will play a limiting role in determining the magnitude of the force required to completely stretch the molecule, since helices seem to unfold at much lower forces. We hope that further studies on similar molecules using SMD and AFM experiments will test this hypothesis. This result might be potentially useful for protein folding and unfolding studies, where force required for unfolding of the loop regions may serve to predict the force required to mechanically stretch the protein.

4 Conclusion

Using SMD simulations, we predict the most flexible and the least flexible regions of α-actinin rod domain by carrying out analysis on all possible hexamer segments in the protein. We observe that the loops and the regions adjacent to loops are the most rigid part of the protein, whereas the most flexible regions are helical. We also address the flexibility of the helical linker, by comparing it to other segments of equal length in the protein. We predict that the helical linker is more flexible than the loops and its flexibility is comparable to the most flexible regions of the protein. However, our results suggest that is not completely flexible or disordered under small mechanical perturbations (< 50 pN). We believe that these results will have a significant influence in our understanding of the flexibility of the α-actinin rod do-


a)
5 pN
b)
10 pN
c)
20 pN
d)
30 pN
e)
40 pN
f)
50 pN
Percent Extension
Hexamer Sequence Starting Residue Number

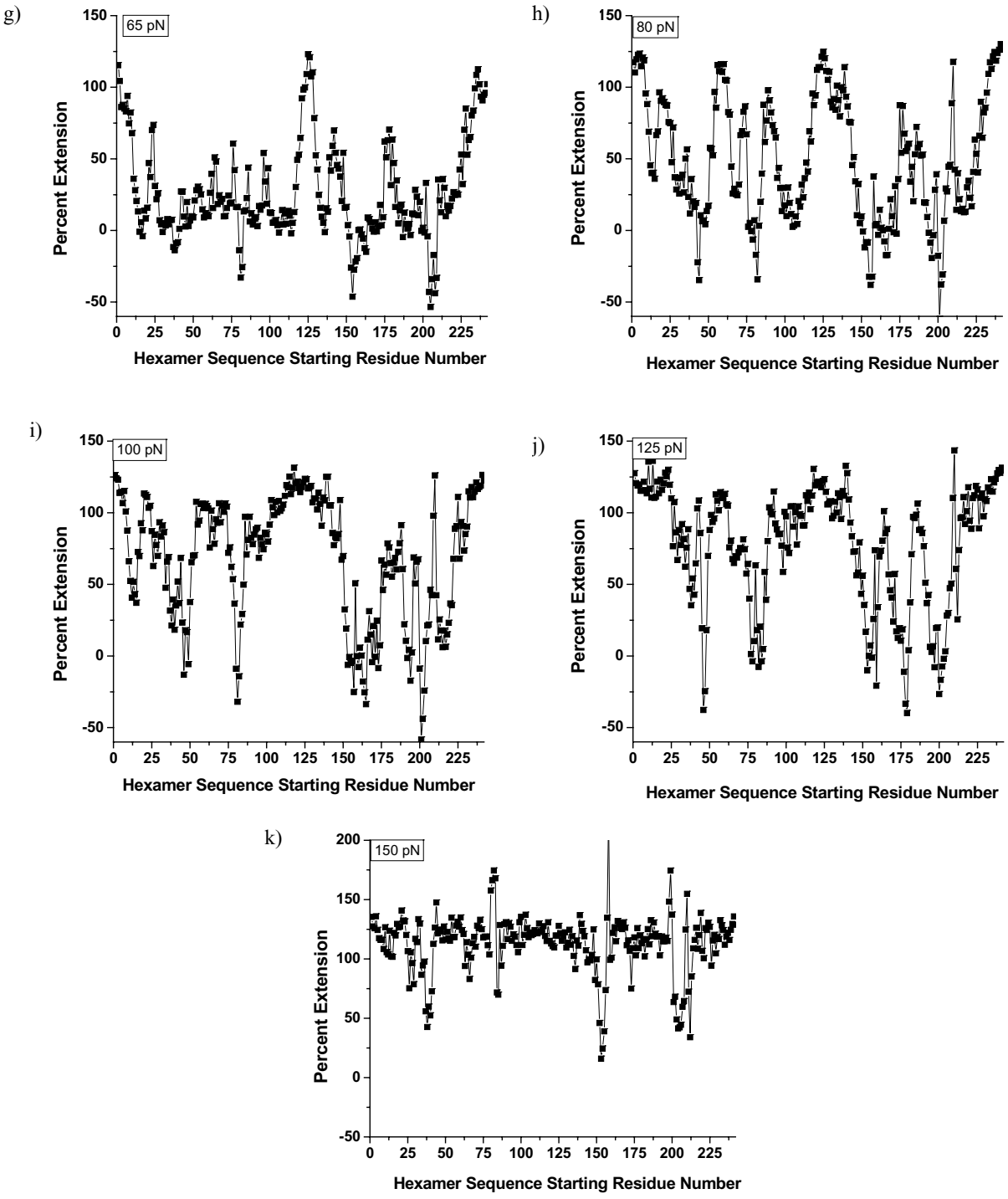


Figure 5 : Percent extension of all possible hexamers in the protein as a function of external force. Percent extension is computed by calculating the average extension of each hexamer in the last 100ps of the forced stretching of the protein and comparing it with the end-to-end distance of the hexamer in the native state. The x-Axis number corresponds the first residue in the segment, e.g number 22 corresponds to the hexamer segment containing amino acids 22-27 and number 49 corresponds to hexamer segment containing amino acids 49-54.
a)5 pN b) 10 pN c) 20 pN d) 20 pN f) 50 pN g) 65 pN h) 90 pN i) 100 pN j) 125 pN k) 150 pN

Supplemental Table S-I. Flexibility ranking of all hexamer segments from 16-230.

Segment Starting Residue	Rank	Segment Starting Residue	Rank	Segment Starting Residue	Rank
16	51	88	73	159	186
17	88	89	59	160	131
18	24	90	66	161	149
19	30	91	114	162	140
20	20	92	36	163	196
21	1	93	9	164	127
22	5	94	58	165	130
23	2	95	70	166	124
24	26	96	17	167	138
25	46	97	62	168	103
26	175	98	119	169	144
27	93	99	60	170	194
28	180	100	61	171	121
29	192	101	183	172	146
30	120	102	139	173	203
31	137	103	84	174	145
32	31	104	101	175	106
33	95	105	135	176	159
34	134	106	128	177	105
35	147	107	164	178	181
36	92	108	78	179	189
37	177	109	107	180	188
38	200	110	108	181	116
39	154	111	109	182	152
40	182	112	64	183	96
41	167	113	81	184	123
42	82	114	142	185	169
43	155	115	43	186	45
44	158	116	110	187	168
45	104	117	112	188	122
46	99	118	11	189	151
47	97	119	54	190	166
48	125	120	16	191	115
49	86	121	55	192	207
50	67	122	44	193	161
51	29	123	6	194	174
52	15	124	35	195	176
53	14	125	18	196	160
54	3	126	10	197	206
55	12	127	65	198	85
56	33	128	34	199	136
57	22	129	56	200	213

58	7	130	57	201	212
59	38	131	48	202	163
60	19	132	75	203	178
61	25	133	173	204	185
62	39	134	148	205	210
63	80	135	117	206	205
64	63	136	153	207	170
65	79	137	32	208	199
66	72	138	87	209	171
67	90	139	8	210	91
68	40	140	4	211	195
69	37	141	49	212	208
70	77	142	74	213	83
71	41	143	100	214	129
72	13	144	165	215	162
73	21	145	141	216	98
74	28	146	102	217	202
75	76	147	157	218	132
76	53	148	23	219	179
77	52	149	126	220	184
78	172	150	143	221	198
79	197	151	187	222	94
80	150	152	201	223	68
81	193	153	215	224	47
82	191	154	214	225	27
83	42	155	211	226	111
84	89	156	209	227	71
85	133	157	204	228	50
86	69	158	118	229	113
87	156			230	190

main, and will improve our knowledge of its function. In addition, we hope that our results will also improve the accuracy of the models that assume the helices as beads connected by completely flexible linkers. Analyzing the amino acid composition of the most flexible regions of the protein provides an interesting insight into the amino acid composition of the most flexible and the most rigid regions of the protein. We observe that the most flexible regions are rich in Ala, Glu and Leu, residues which are either absent or rarely present in the most rigid regions. On the other hand, Ser, Ile and Val are almost always present in the most rigid regions and almost always absent in the most flexible regions. This shows that the not only the secondary structure, but also the amino acid composition of a given region makes it flexible or rigid under external mechanical stress.

Finally, we also suggest that the loop regions of a protein, which is primarily composed of α-helices and loops, unfold at the same forces as the molecule itself, and therefore the mechanical unfolding of these regions can be used to predict the force required to unfold the protein.

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