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PROTEIN STRUCTURE TO FUNCTION *VIA* DYNAMICS

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Abstract: Protein folding, binding, catalytic activity and molecular recognition all involve molecular movements, with varying extents. The molecular movements are brought upon *via* flexible regions. Stemming from sequence, a fine tuning of electrostatic and hydrophobic properties of the protein fold determine flexible and rigid regions. Studies show flexible regions usually lack electrostatic interactions, such as salt-bridges and hydrogen-bonds, while the rigid regions often have larger number of such electrostatic interactions. Protein flexible regions are not simply an outcome of looser packing or instability, rather they are evolutionally selected. In this review article we highlight the significance of protein flexibilities in folding, binding and function, and their structural and thermodynamic determinants. Our electrostatic calculations and molecular dynamic simulations on an antibody-antigen complex further illustrate the importance of protein flexibilities in binding and function.

Protein flexibilities: An essence to folding, binding and function

Flexible regions are central to both protein folding and function.

Flexible regions *Flexible regions*

Sequence ⟶ Structure (Fold) ⟶ Function

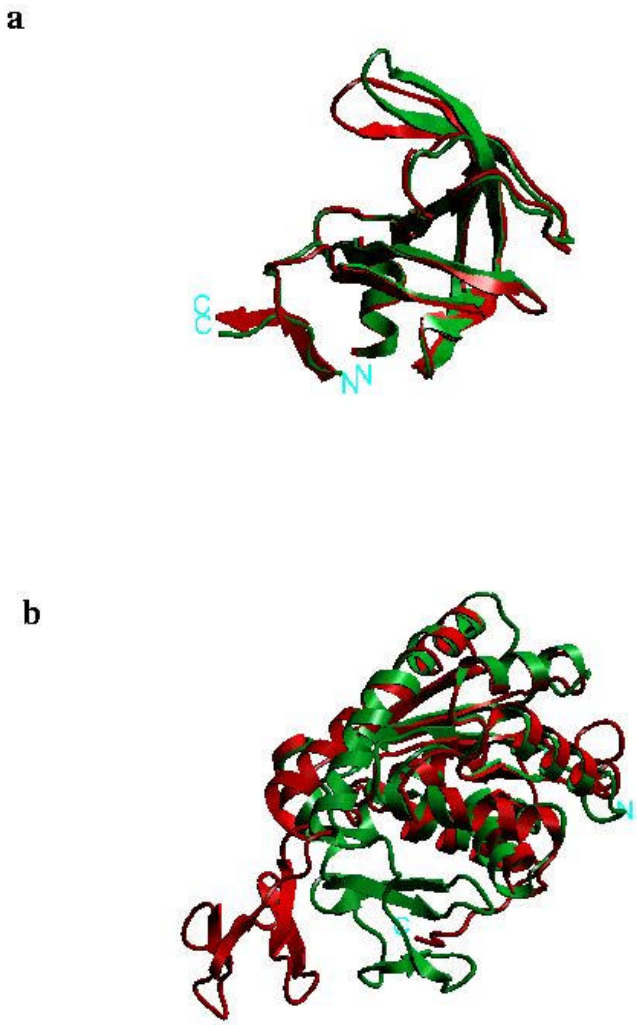

Figure 1. Protein movements for function. Superimpositioning of 'closed' and 'open' conformations in (a) HIV Proteinase (Red-closed; Green-open); (b) Adenylate kinase (Green-closed; Red-open). *N* and *C* terminus of proteins are marked. The superimpositioning is performed using Geometric Hashing program [61].

Protein flexible regions allow the precise movement in thousands of atomic co-ordinates to perform function. Several examples of bona fide protein movement cases have been reported in the literature. Such examples include flap movements in retroviral protease, domain movements in T-4 lysozyme, calmodulin and adenylate kinase and fragment movements in lactate dehydrogenase (Figure 1). The links between their movements and function have been established by X-ray crystallography and other experimental observations.

For example, flap movements in retroviral proteinase are conserved throughout the family of aspartyl proteinase. Consisting of a β-hairpin, the flaps at the ceiling of the binding pocket move about 7 Δ between its 'closed' and 'open' conformations [1-3]. The rapid flap movements have been shown by NMR and fluorescence changes. In the case of adenylate kinase a four stranded anti-parallel β-sheet is shown to undergo significant displacement upon substrate binding [4,5]. Calmodulin, T-4 lysozyme, troponin C, lactoferrin and glutamate dehydrogenase are other such bona fide examples, where the movements have been linked with their respective functions. A large number of cases, where two or more conformations have been crystallized, indicate clear movements (reviewed by Gerstein and Krebs-[6]). The extents of movements vary depending on their functional requirements. For example DNA polymerase-β undergoes a very large movement (about 11 Δ to accomodate DNA) as compared to glutamate dehydrogenase (about 0.5 Δ) [7]. 'Domain-swapping' [8], the binding to multiple substrates under different conditions, allosteric regulation, operation of molecular motors and binding cascades all are also due to conformational adaptabilities [7,9,10,11], stemming from flexible regions. Flexibility/rigidity compensations determine protein thermostability. The binding site of a secondary antibody, of high affinity towards its antigen, would consists of flexible and rigid regions, "pre-selected" for their respective roles. Similarly, in hinge-bending type of movement, as seen in adenylate kinase or calmodulin, the flexible hinge-points are selected to allow the motion. "Lock and key" or "induced fit" type of binding are also selected, rather than just an outcome of the structural details. It is clear that the protein movements, whether involving subunits, domains or any secondary structural elements, and their extents are uniquely selected for the respective function. Since fold also relates to function, a particular sequence is evolutionally selected for both structure and function.

Protein flexibilities: An intrinsic property?

Above, we have emphasized that protein flexibilities are their functional requirements. Here we describe that protein flexible regions are present *a priori* in the structure, i.e. they are encoded in their sequences. Flexibility-governed protein folding, misfolding and aggregation all are sequence encoded. Protein function is therefore an outcome of optimized flexibilities, resulting in very precise movements. Proteins acquire their unique 3-D folds *via* flexible regions. A molten globule revealed by folding simulation had same global fold as the native state. The secondary structures were identical, however, the larger flexibility were observed in the turn regions and at chain ends [12]. These inherent flexible regions guide a sequence to acquire a unique 3-D fold. In solution proteins undergo movements of different scales ranging from atomic fluctuations, bond oscillations, side-chain oscillations to hinge- bending movements, helix-coil transitions and folding/unfolding process [13]. NMR spectroscopy and MD simulations have provided insights into these dynamical events [14,15], to perceive their roles in protein function and folding [16,17]. Protein misfolding, as in amyloid formation, or aggregation, takes place when unoptimized flexibility results in misfolded conformations being more stable than the native ones. Single point mutations can cause such unoptimized flexibility. For example, an

amyloidogenic form of human lysozyme differs from the non-amyloidogenic form by a single point mutation. This mutation disrupts a hydrogen-bond network [18], making the mutant form less stable and more prone to misfold. Similarly transthyretin, gelsolin and cystatin C all form amyloid, or become more prone to undergo such conformational changes, due to naturally occurring point mutations at crucial positions. On the other hand in healthy protein conformations the extent of flexibilities are balanced. They have inherent flexible regions [19]. Any structural perturbation due to non-lethal point mutations is accommodated *via* these flexible regions [19].

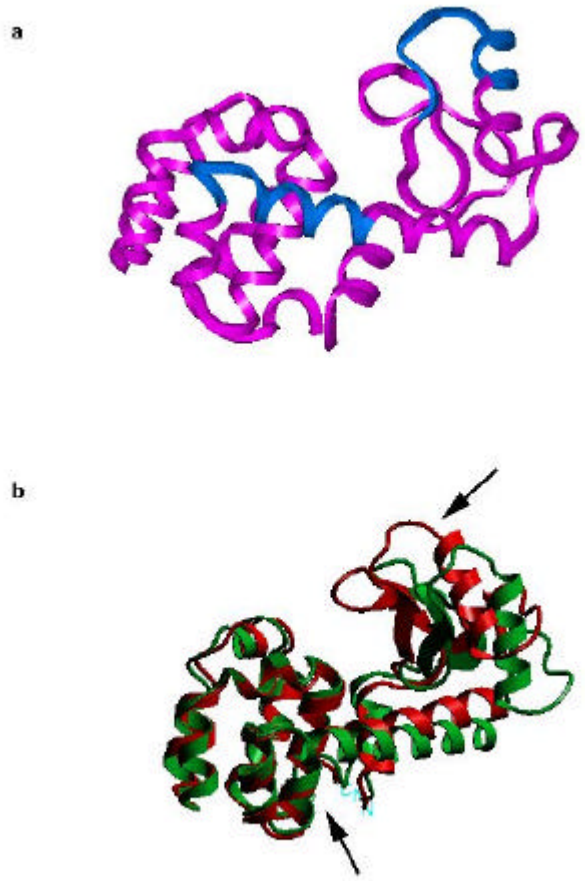

Figure 2. Protein flexibility required for function. (a) The flexible regions (blue) of T-4 lysozyme. Flexibility assignment is from Sinha and Nussinov (2001); (b) Superimposed 'Closed' (red) and 'Open' (green) conformations of T-4 lysozyme. The arrow shows the functional hinge points. The hinge points are taken from the database of molecular movements [6]. Superpositioning is performed using Geometric Hashing program [61].

Flexibilities are essential for both structural integrity and functional properties. Mutating nine residues of T-4 lysozyme increased its stability, but had an adverse effect on the activity of the enzyme [20]. Luque and Freire [21] show, using the HIV proteinase example, that the large conformational entropy of binding would result in lower affinity. The straightforward way to minimize the conformational entropy is if the binding site is relatively unstable (or flexible), and occupies a shallow energy minimum [21]. The barrier between bound and unbound states will be small, so the unbound state can easily flip to bound upon ligand encounter. Protein flexible regions fall on functional hinge-points, which allow protein movements for binding and function [22]. Figure 2 shows such a correlation between flexible regions and functional hinge points, where the flexible regions of T-4 lysozyme are in and around hinges required for function. The propagation of conformational changes over large distances in allosteric proteins, and the conformational changes at remote positions due to substrates at active sites [23-26], are *via* flexible regions. Flexible regions play key roles in the operation of molecular motors. A microtubule-based motor enzyme kinesin has an *N*-terminal catalytic motor domain, a stalk and a globular *C*-terminus. A flexible hinge region present in the stalk plays an essential role in the operation of motor. Deletion and truncation of the hinge region reduce the motor speed [27].

The thermophilic proteins are structurally more rigid than their mesophilic counterparts at mesophilic temperatures. Modulation of electrostatic [28,29] and hydrophobic properties restrict the flexibilities and enhance the compactness [30] in these proteins. At their physiological temperatures they have marginal stability and increased flexibility, essential to their function. Thermophilic proteins were proposed to acquire structural rigidity by increase in the number of ion-pairs and their energetic optimizations [31-34]. Freire [35] has also shown that the changes at a local site can affect structurally distant regions. Thus protein sequences are evolved such that the following unique fold will consist of 'flexible' and 'rigid' regions. The extent, and proportion, of flexibility or rigidity, would indeed depend on the functional requirements.

Structural and thermodynamic determinants: Electrostatics and Hydrophobicities

Charged and polar atoms upon protein folding overcome desolvation penalties by forming electrostatic interactions among themselves. Non-polar atoms repel water and are sequestered in the protein interior, due to their hydrophobic nature. Electrostatics either act locally, through salt-bridges or hydrogen-bonds, or act globally by defining the over-all electrostatic environment of the protein. These two properties of the protein, electrostatic and hydrophobicity, or their compensations, mainly determine the regions of plasticity or tightness in the protein fold, and the extent of conformational flexibility a protein can adopt during binding and function. Electrostatics have been linked with specificity or conformational rigidity. Hydrophobicity, on the other hand, allows flexibilities or conformational adjustments. Electrostatic interactions, mainly salt-bridges and hydrogen-bonds, are shown to be present in conformationally rigid regions [36], or where the binding is more specific [37-39] or is of high affinity [40,41]. They play major roles in molecular recognitions [42]. Absence of electrostatic interactions, or their weak electrostatic strengths, is shown to allow protein movements for function [7,43].

Thermophilic proteins have higher number of salt-bridges than their mesophilic counter-parts [44]. The antibody more specific towards its antigen not only has higher number short range electrostatic interactions at its binding site, but also has the binding site salt-bridges with higher electrostatic free energy contributions [39]. High electrostatic complementarity between Barnase and Barstar results in very tight and specific binding between these two proteins [45]. Their rapid associations are electrostatically assisted [46]. On the other hand electrostatic interactions are avoided in conformationally flexible parts [7,10,47].

However, non-polar buried surface area, or hydrophobicity, can be quite extensive in conformationally flexible parts [7,10]. The extent of hydrophobicity is linked to the extent of movements [7]. The cross-reactive or a non-specific binding involves larger hydrophobic residues [48], and flexible regions required for function require some threshold level of hydrophobicity [49]. Therefore, both protein folding and binding require fine tuning of electrostatic and hydrophobic properties. It allows well balanced movements for function, but disallows misfolding. The protein-protein associations, or a binding of an affinity matured antibody, require well tuned electrostatic/hydrophobic compensations. The extent of electrostatics and hydrophobicity would in turn determine the binding mechanism, for instance 'lock and key' versus 'induced fit'. These compensations would be inherent to the protein fold, depending on the extent of flexibility/rigidity required for the function.

Antibody-antigen binding: An example

Above we have discussed the involvement of flexibility in protein folding and binding. Here we address entailed flexibilities in the formation of large protein-protein complexes. Antibody-protein antigen complexes have long served as a model to understand the fundamentals of molecular associations or recognitions [42,50-52]. It has long been perceived that the binding can be either "lock and key" or "induced fit" type. This is in-general true for the formation of protein-ligand or antibody-hapten complexes, which bury smaller surface areas upon complex formation. However, in the formation of large protein-protein complexes, like antibody-hen egg white lysozyme (HEL) complex, which bury much larger surface area, 1200-2000 Δ^2, the binding would be partially "induced fit" and partially "lock and key". Some regions at the binding surface would be rigid, undergoing "lock and key" fit, while the other regions would be flexible, undergoing "induced fit". In both, small and large complexes, the flexible and rigid regions are selected for function. Comparison of a complexed anti HEL antibody with its un-complexed forms reveal that for the heavy-chain the root mean square deviations were greater and more variable, suggesting that the binding site flexible regions are in heavy chain. The significant differences were found in CDR-H2 and CDR-H3 (deviations upto 2.7 Δ and 1.9 Δ, respectively) [53]. In five other anti-HEL antibodies the structural rearrangements upon binding occur as < 3 Δ movements in CDR-loops, side-chain rearrangements and changes in the relative orientations of the V_H and V_L domains. The complex formation between large protein-protein partners, like antibody-protein antigen and proteinase-proteinase inhibitor, would inherently involve different extents of movements at different locations of the binding site. Freire [35] shows that anti-hen egg white lysozyme antibody D1.3 binding effects are propagated

to the remote locations from the binding epitope, suggesting regions of flexibility/rigidity in an uncomplexed binding site of lysozyme or D1.3, or both. Similarly, amide exchange kinetics show that lysozyme binding to an antibody purturbed a few distantly located residues [54,55]. For antibody-antigen associations these regions are selected during affinity maturation.

A short molecular dynamics simulation (200 ps) of an anti-HEL antibody, HyHEL10(HH10), complexed with HEL, HH10-HEL, reveals that the binding site of this antibody contains more and less flexible, or flexible and rigid, regions. This affinity matured monoclonal antibody has a very high affinity towards its antigen [38,48]. Thus, these rigid and flexible regions are optimized for high affinity binding. They are inherent to its structure and are predisposed *via* structural and thermodynamic forces, stemming from its sequence. Figure 3 shows flexible and rigid regions at the binding site of an affinity matured antibody.

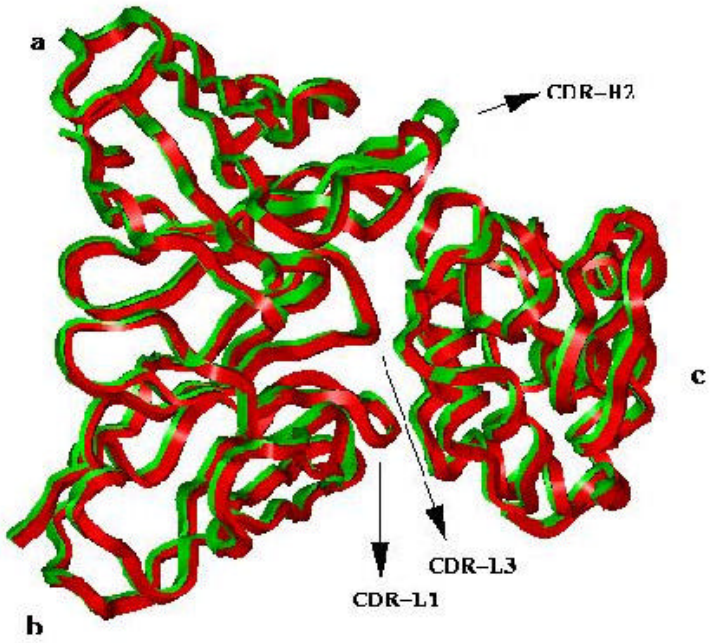

Figure 3. Superpositioning of the complexes of HyHEL10-HEL X-Ray crystal structure (red) and the conformer at the 20 ps time step of the molecular dynamics simulation (green). Regions of higher relative mobilities (CDR-H2, CDR-L3 and CDR-L1) are shown with arrows. a, b, and c are antibody Heavy chain, Light chain and antigen Hen Egg White Lysozyme, respectively.

Table 1. Revealed Salt-bridges of HH10-HEL complex during 200 ps MD simulation. Salt-bridges present in X-ray crystal structure and in the MD conformers collected at the regular time intervals. Salt-bridges are shown by three letter code residue names followed by the position. The chain identifications are shown in subscript; H: Heavy chain; L: Light chain; Y:Lysozyme. Intra and inter-molecular salt-bridges are marked. MD calculations were performed *in vacuo*, using the C-DISCOVER module of INSIGHT II, in NVT (canonical) ensemble. The system was subjected for equilibration for 100 ps, before the data collection. The system included the complete interface residues of HH10 and HEL along with bordering residues within about 30.00 Δ distance on all sides.

Conformer	Salt bridge	Intramolecular	Inter-molecular
X-ray structure	$Asp99_H$-$His34_L$	T	
	$Asp99_H$-$Lys49_L$	T	
110ps	$Asp99_H$-$Lys49_L$	T	
	$Asp101_H$-$Lys49_L$	T	
	$Lys13_Y$-$Asp18_Y$	T	
120ps	$Asp32_H$-$Lys97_Y$		T
	$Asp99_H$-$Lys49_L$	T	
	$Asp101_H$-$Lys49_L$	T	
	$Asp48_Y$-$Arg61_Y$	T	
130ps	$Asp32_H$-$Lys97_Y$		T
	$Asp99_H$-$Lys49_L$	T	
	$Asp101_H$-$Lys49_L$	T	
	$Lys13_Y$-$Asp18_Y$	T	
	$Asp48_Y$-$Arg61_Y$	T	
140ps	$Asp32_H$-$Lys97_Y$		T
	$Asp99_H$-$Lys49_L$	T	
	$Asp101_H$-$Lys49_L$	T	
	$Lys13_Y$-$Asp18_Y$	T	
150ps	$Asp101_H$-$Lys49_L$	T	
	$Lys13_Y$-$Asp18_Y$	T	
160ps	$Asp32_H$-$Lys97_Y$		T
	$Asp99_H$-$Lys49_L$	T	
	$Asp101_H$-$Lys49_L$	T	
	$Lys13_Y$-$Asp18_Y$	T	
170ps	$Asp32_H$-$Lys97_Y$		T
	$Asp101_H$-$Lys49_L$	T	
	$Lys13_Y$-$Asp18_Y$	T	
180ps	$Asp32_H$-$Lys97_Y$		T
	$Asp99_H$-$Lys49_L$	T	
	$Asp101_H$-$Lys49_L$	T	
	$Asp48_Y$-$Arg61_Y$	T	
190ps	-		
200ps	$Asp99_H$-$His34_L$	T	
	$Asp99_H$-$Lys49_L$	T	

In solution, a protein exists in a range of conformational isomers. Around the native state low kinetic barriers separate these isomers. The non-bonded interactions, like salt-bridges and hydrogen-bonds, thus are formed and broken, depending upon the side-chain fluctuations in the solution. An analysis of NMR conformers show such behavior [56]. The population of a particular conformer, or a molecular interaction, however, would depend on the functional requirements, under the native conditions. An important conformer, or a molecular interaction, would have a higher population time in solution. This means that an X-ray crystal structure only represents one among several conformations around the native state. This "snap-shot" conformation during the crystallization is selected depending on the crystallization conditions. This implies that an X-ray structure may not provide the complete picture of all the molecular interactions, or reveal all the molecular interaction "hot-spots". Our MD simulation of HH10-HEL complex reveals many important inter and binding site intra-molecular salt-bridges, not shown in its X-ray crystal structure. The importance of these molecular interactions are based on the following: i) These interactions are present in three other antibody-antigen complexes, belonging to the same family [39]; ii) All the revealed interactions involve "hot-spot" epitope residues, experimentally shown to contribute significantly towards binding [38,48,57]; iii) The revealed interactions recur during simulation. Table 1 shows the revealed salt-bridges during the MD simulation of HH10-HEL complexes. The revealed inter-molecular salt bridge (Table 1), $Asp32_H$-$Lys97_Y$, involves an "hot-spot" epitope residue [57], shown to contribute the most, among all the epitope residues, towards HH10-HEL complex formation, both experimentally and computationally [39]. This salt-bridge has also been shown to be significantly stabilizing towards folding and binding in the complexes belonging to the same families [39]. The biological importance of these molecular interactions is evident. In order to see a complete picture of all molecular interactions, especially those which are biologically important, the range of conformations should be evaluated. Obviously this can be straightforwardly achieved by performing MD simulations.

CONCLUSIONS

In this review we summarize how flexibility or the property of conformational plasticity is involved in every aspect of protein structure and function. They are evolutionally selected *via* sequences. The rigid regions are important in providing the framework. The extent of flexibility depends on the functional requirements and physiological needs. For example: larger domain movements in calmodulin [58] versus small fragment movements of triosephosphate isomerase [59]; hinge movement versus shear movement [6]; "lock and key" type of binding versus "induced fit" binding; stability of a thermophilic protein [60] versus mesophilic protein [44]; the flipping of the secondary structural elements versus the whole domain in domain swapping events [8] all are optimally selected during evolution for their respective function. For example- calmodulin undergoes large conformational changes upon Ca^{++} binding, which triggers its associations with target proteins. An 11 residue loop in triosephosphate isomerase closes the active site upon substrate binding. The difference in movements in the two cases are required to perform different kinds of functions. On the other hand unoptimized flexibilities

may lead to protein aggregation, or misfolding, like conformational changes in prion protein [10]. This usually results due to mutations or changes in physical conditions, like pH, temperature, concentrations etc. Electrostatics and hydrophobic properties mainly determine regions and extents of protein flexibilities. A very specific binding requires larger, and stronger, electrostatic interactions [39], and flexible regions lack electrostatic interactions, where the extent of non-polar buried surface area may determine the extent of movements [7]. Our MD simulations on antibody-antigen complexes corroborate the inherent nature of protein flexible regions. A similar study shows that the binding site of the light chain and heavy chain of an affinity matured antibody consists of "pre-selected" flexible and rigid regions, for their respective roles in high affinity binding (Sinha and Smith-Gill, unpublished results). The knowledge of the determinants of protein flexibilities, or flexibility/rigidity compensations, is essential to perceive the fundamentals of protein folding, and its relation to function. Such studies also have applications in research areas of molecular recognition, protein-protein interaction, antibody affinity maturation and drug design.

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