Electrostatics in Protein Binding and Function

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Abstract: Protein electrostatic properties stem from the proportion and distribution of polar and charged residues. Polar and charged residues regulate the electrostatic properties by forming short-range interactions, like salt-bridges and hydrogen-bonds, and by defining the over-all electrostatic environment in the protein. Electrostatics play a major role in defining the mechanisms of protein-protein complex formation, molecular recognitions, thermal stabilities, conformational adaptabilities and protein movements. For example:- Functional hinges, or flexible regions of the protein, lack short-range electrostatic interactions; Thermophilic proteins have higher electrostatic interactions than their mesophilic counter parts; Increase in binding specificity and affinity involve optimization of electrostatics; High affinity antibodies have higher, and stronger, electrostatic interactions with their antigens; Rigid parts of proteins have higher and stronger electrostatic interactions.

In this review we address the significance of electrostatics in protein folding, binding and function. We discuss that the electrostatic properties are evolutionally selected by a protein to perform an specific function. We also provide bona fide examples to illustrate this. Additionally, using continuum electrostatic and molecular dynamics approaches we show that the "hot-spot" inter-molecular interactions in a very specific antibody-antigen binding are mainly established through charged residues. These "hot-spot" molecular interactions stay intact even during high temperature molecular dynamics simulations, while the other inter-molecular interactions, of lesser functional significance, disappear. This further corroborates the significance of charge-charge interactions in defining binding mechanisms. High affinity binding frequently involves "electrostatic steering". The forces emerge from over-all electrostatic complementarities and by the formation of charged and polar interactions. We demonstrate that although the high affinity binding of barnase-barstar and anti-hen egg white lysozyme (HEL) antibody-HEL complexes involve different molecular mechanisms, it is electrostatically regulated in both the cases. These observations, and several other studies, suggest that a fine tuning of local and global electrostatic properties are essential for protein binding and function.

PROTEIN PROPERTIES: THE ROLES OF ELECTROSTATICS

Innumerable experimental and theoretical studies have established connections between protein electrostatic properties and function [1-6]. It is generally believed that a sequence acquires a globular form in micro sec to milli sec time scale due to its hydrophobic interactions, or due to hydrophobic side-chains escaping from water. In the interior of the protein the back-bone polar atoms compensate their charge desolvation by forming favorable electrostatic interactions as main chain-main chain hydrogen-bonds (H-bonds), resulting in the formation of secondary structures. The specificity of the tertiary structure, binding and unique function stems from spatial arrangement of polar and charge atoms, and by formation of electrostatic interactions, mainly salt-bridges and main chain-main chain, main chain-side chain and side chain-side chain H-bonds (Fig. **1**). For instance the high specificity and selectivity of antibodies toward protein antigens is mainly through electrostatic forces [7]; The driving force towards thermostability is through the optimization of electrostatic interactions by increasing the number of salt-bridges [8-10], with more favorable electrostatic interactions in thermophilic proteins [11, 12]; Protein recognition sites on average contain 10-inter-molecular H-bonds [13], and protein interfaces are richer in both charged and polar residues, compared to protein cores [14, 15]; Low affinity of a haemoglobin mutant is linked to the electrostatic and steric effects of the introduced charge side-chain [16]; The presence and absence of salt-bridges define the tense (T) and relaxed (R) states of hemoglobin [17]; Consistently presence and absence of a inter-subunit salt-bridge is proposed to define conformational distribution between R and T states of pyruvate kinase [18].

Computational and experimental analyses have shown that salt-bridges can be stabilizing [19-21] or destabilizing [22, 23]. Both ways they play important structural and functional roles. Stabilizing salt-bridges rigidify local regions for better fit due to functional requirements [20]. A destabilizing salt-bridge would provide conformational specificity for the fold or function [23]. The number of H-bonds and salt-bridges, and stabilizing and destabilizing nature of salt-bridges play major roles in defining the extent

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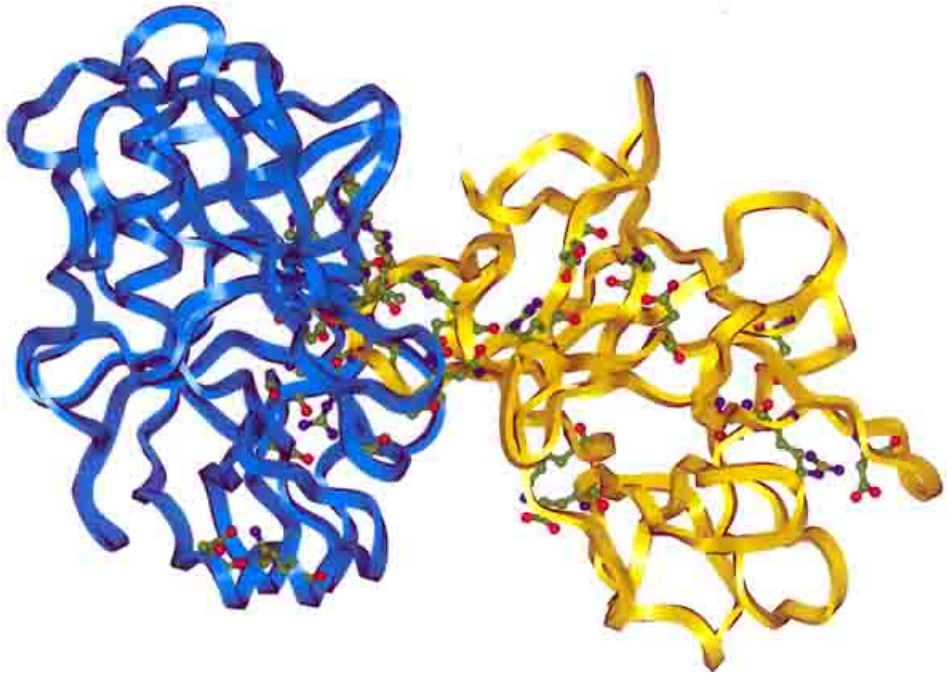

Fig. (1). Salt-bridges and inter-molecular hydrogen-bonds in trypsin-soyabean inhibitor complex (Pdb id:1avw). Salt-bridges and H-bonds are shown with their side-chains, colored by atom type. Trypsin and soyabean inhibitor are shown in cyan and yellow colors, respectively. The picture is created using InsightII.

of flexibility and rigidity in proteins [24]. Both flexible and rigid regions are equally important for function. The rigid portions provide frame-work, while flexible regions are necessary for protein movements, for function [24-26]. A destabilizing salt-bridge breaks while a stabilizing salt-bridge network remains intact during the transition from 'closed' to 'open' conformation in T-4 lysozyme [24]. This stabilizing network lies very close to the hinge, while the destabilizing salt-bridge joins the two domains, which are required to undergo large scale separation during the transition. The evolutionarily selected electrostatic strength of these salt-bridges allow, or disallow, the movements, or may govern the movement extent [24].

Continuum electrostatic calculations and molecular dynamics (MD) simulations show that electrostatics is a major determinant of antibody-antigen binding specificity and cross reactivity [27]. The "hot-spot" inter-molecular interactions in a very specific antibody-antigen binding are mainly established through charged residues. Here we show that important inter-molecular charge-charge interactions, involving "hot-spot" epitope and paratope residues, stay intact even during high temperature molecular dynamics simulations, when the over-all antibody-protein antigen binding interface is significantly disrupted. Inter-molecular H-bonds at 100^{o}C involve experimentally shown "hot-spot" epitope and paratope residues. This further highlights the structural/functional importance of polar/charge interactions in high affinity binding. The finding also suggests that the significant contributions towards the high affinity binding are through short range electrostatic interactions. "Electrostatic steering" is generally believed to involve long range interactions, which enhance the diffusion limited collision. However, electrostatics can also act in the stabilization of the initial encounter complex by forming inter-molecular salt-bridges and H-bonds [27]. Continuum electrostatic calculations show that high affinity binding in barnase-barstar and antibody-hen egg white lysozyme (HEL) complex mainly involve electrostatic forces. In barnase-barstar complex the electrostatic forces act as long-range, and possibly later on by forming inter-molecular salt-bridges and H-bonds. We show that in the case of an antibody-HEL complex formation electrostatic forces act as short range interactions, but not as long range effects, and stabilize the encounter complex. The study suggests that although the mechanism varies, the high affinity binding in both cases is electrostatically governed.

THE HINGE-BENDING MOTIONS

The presence of inherent flexible and rigid regions in proteins allow the movements for their function [24-26]. Proteins undergo mainly two types of motions: Hinge and shear [28]. In shear motions the structural units slide with respect to each other, maintaining the interface. In hinge motions a fragment, domain or a subunit moves more or less as a rigid body from the rest of the protein, and thus breaking the interface. Hinge-bending involves large movements (1.0 Å- 14.00 Å). Moving fragments, domains and subunits have an insignificant number of H-bonds connecting them to the rest of the protein. They have either none or one inter-facial salt-bridge [24]. The electrostatic strengths of the inter-facial salt-bridges are small, compared to the salt-bridges found in

other parts of the protein. These observations imply that the selected electrostatic interactions are such that the energy barriers of transitions between 'close' and 'open' conformations are low. Upon binding these proteins can easily convert to their closed forms.

DOMAIN SWAPPING AND AMYLOID FORMATION

Eisenberg and colleague described protein dimerization and oligomerization by 'domain-swapping' events. In domain swapping a secondary structural element or a whole domain are exchanged between the two or more sister monomers [29, 30]. These domains or structural units are connected with the rest of the protein through long coils. Systematic analysis of domain-swapping and amyloidogenic proteins show that amyloid fibril formation can be explained by 'domain swapping'-like events [31]. Other groups have proposed domain swapping as a general mechanism for amyloid formation [32-34]. Finding that amyloidogenic cystatin C forms a domain swapped dimer [33,35] corroborated this hypothesis, and any change in the physiological conditions or mutations lead to amyloid formation, where domain swapped dimer is the most populated species [33]. As in domain swapping cases, in amyloidogenic proteins a potential motif was proposed to swing out from the rest of the structure [31]. The only difference between domain swapped cases and amyloidogenic proteins was that while in domain swapping cases the swapped domain can be a domain or a secondary structural element, such as β-strand or an α-helix, but never a β-hairpin motif, the potential motif in amyloidogenic proteins was always a β-hairpin. The β-hairpin motif was connected to the rest of the protein *via* a long coil. The swapping domain and the potential motif have insignificant numbers of H-bonds connecting them to the rest of the protein. There were either none or one salt-bridge present between the motif and the rest of the protein. The near absence of short range electrostatic interactions allows swinging out the domain or a β-hairpin [31]. Furthermore polarity, H-bonding and packing show that the inter-domain interfaces are more similar to protein surfaces than cores. This would allow domain to swing out and swap during oligomerization [36]. Based on this it was proposed that the β-hairpin motif swings out and stacks with a similar motif from another monomer in a growing amyloid fibril. One way of engineering amyloidogenic proteins to more stable proteins would be engineering short range electrostatic interactions at the interfaces of the potential motifs.

PROTEIN STABILITY

Proteins are evolutionarily selected for the optimum compromise between flexibility and stability. Too much of stability would hamper protein function [37], while too little may result in protein misfolding [31]. The free energy of protein stabilization is only a few kcal/mol, equivalent to a few stabilizing non-bonded inter-molecular interactions. However, proteins are modulated towards higher stability in order to survive under extreme environmental conditions, like high temperature, very low pH and high salt. Higher number of charged residues [38], short range non-bonded interactions [39], such as H-bonds and salt-bridges and higher electrostatic strengths of ion-pairs [11, 12] are shown to be the reasons for enhanced stabilities in the proteins surviving under extreme environmental conditions. The higher kinetic barrier towards unfolding of rubredoxin from hyperthermophilic *Pyrococcus Furiosis* compared to from mesophilic *Clostridium pasteurianum* was proposed due to fixing of the protein at crucial positions, *via* ion-pairs [40]. Sequence comparison of a cold shock protein CspB from mesophilic-*Bacillus subtilis*, thermophilic- *Bacillus caldolyticus* and hyperthermophilic-*Thermotoga maritima* show that the thermophilic stabilization is not only caused by the optimizations of existing charge-charge interactions but also by the introduction of new favorable charge-charge interactions [41]. Similarly, a combinatorial mutagenesis experiment show that the thermostability of a cytochrome P450 from *Sulfolobus solfataricus* were due to surface charge residues involved in the formation of salt-bridges [42].

BINDING SPECIFICITY AND AFFINITY

High affinity and a very specific binding involves polar and charge interactions. While hydrophobicity and the lack of electrostatic interactions allows conformational flexibilities [24, 31]. Binding interfaces have larger numbers of charged residues compared to protein cores [14] (Fig. **2**). The enzyme inhibitor complexes have been shown to have null mean hydrophobicities, and the interactions are mainly through polar and charged residues [14]. The charge complementarity leads to specific binding of high affinity [43-45]. High affinity binding of proteinase-proteinase inhibitor complexes and antibody-protein antigen complexes are through polar and charge interactions [46-49], where only few a charge-charge interactions actually contribute most to free energy of binding [50-52]. In an independent analysis of protein-protein complexes alanine scanning showed that in most of the cases mutations of charged and polar residues destabilized the complex [53]. Faster association rate constants, than estimated due to simple diffusion, are proposed due to favorable electrostatic inter-molecular forces between the proteins [54-57]. A high affinity antibody, very specific towards its antigen, has larger number of very stabilizing salt-bridges at its binding site, compared to a structurally related antibody, less specific towards the same antigen epitope [27]. Fig. (**3**) show salt-bridges and a very stabilizing salt-bridge network at the binding site. All of these observations, and many others, corroborate the fact that electrostatic forces determine specificity and affinity of protein-protein binding, whether it is a formation of a small complex such as that of hirudin-thrombin or a formation of a large protein -protein complex, like antibody-HEL. The strength of electrostatic interactions at the binding site in turn determines the extent of specificity; higher strength leads to more geometrically constrained binding [27]. Fig. (**2**) shows the distribution of charge and polar residues at the protein-protein interface.

LIGAND BINDING AND ENZYME CATALYSIS

Electrostatics play a major role in enzyme catalysis [58-62]. Electrostatics have been shown to stabilize the transition

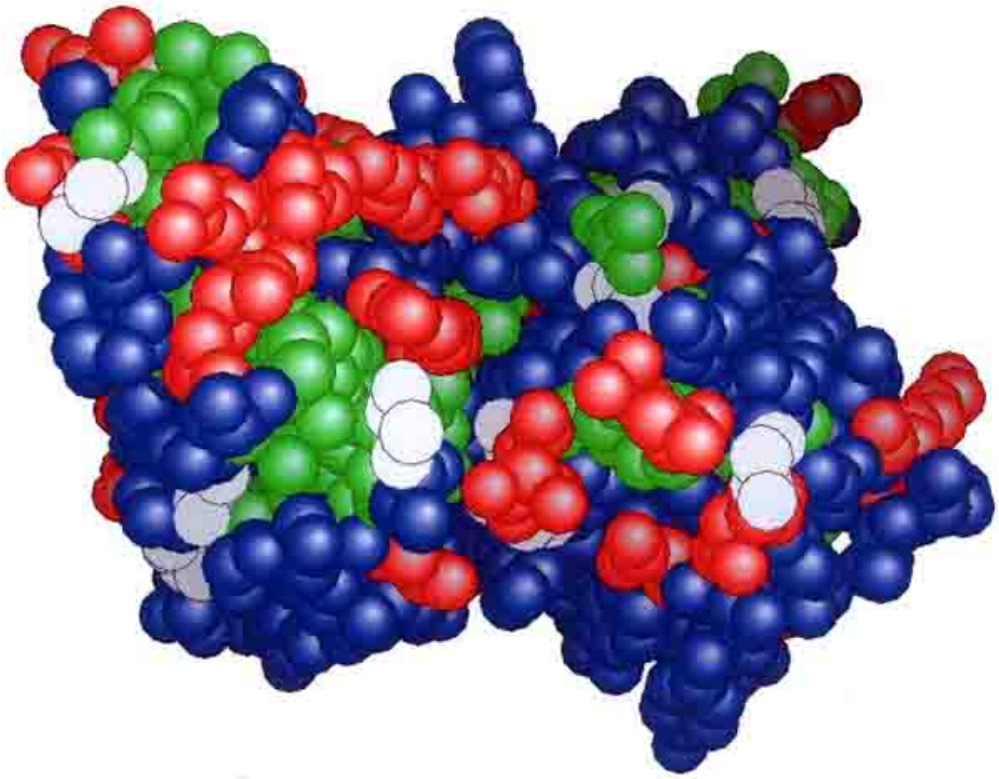

Fig. (2). Binding site hen egg white lysozyme antibody HyHEL63 (Pdb id: 1dqj). Hydrophobic, polar and charged residues are shown in green, blue and red colors. Picture is generated using GRASP [112].

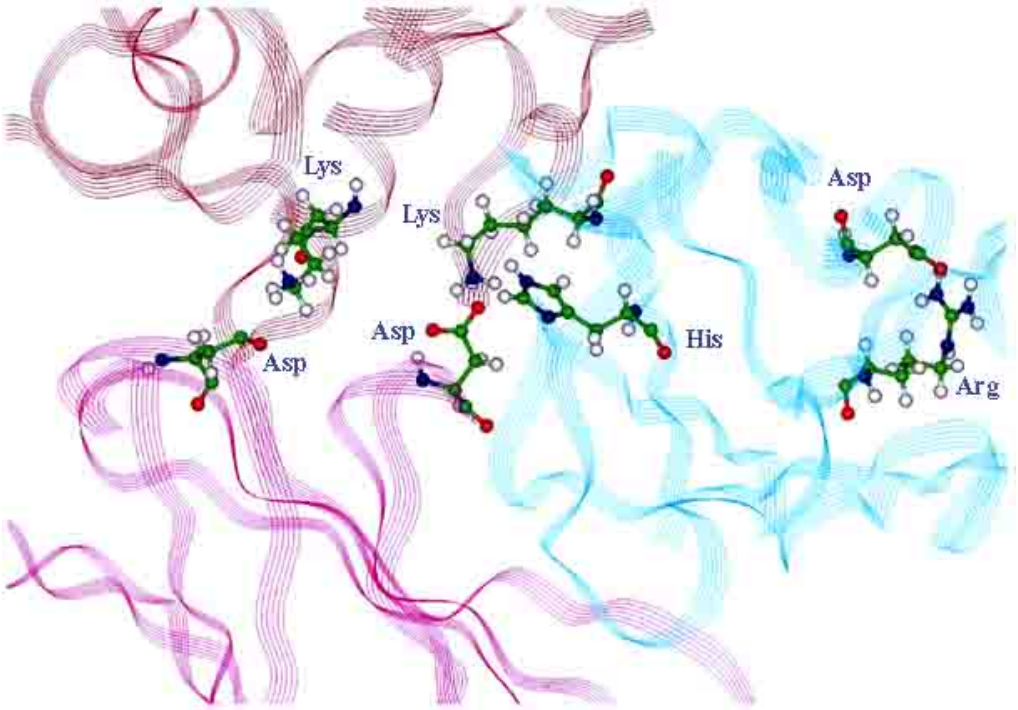

Fig. (3). Binding site salt-bridges and a very stabilizing salt-bridge network at protein-protein interface. Salt-bridges at the binding site of anti-heg egg white (HEL) lysozyme antibody HyHEL10-HEL compex. Light chain, Heavy chain and lysozyme are shown in cyan, megenta and brown colors, respectively. The salt-bridge network formed from Asp, Lys and His residues contributes -6.28 Kcal/mol towards folding [27]. The salt-bridges are shown with their side-chains on, colored by atom-types. The picture is created using InsightII. Salt-bridge forming residues are labelled in three letter residue codes.

state of the enzyme-substrate complex. The enzyme binding site consist of charge and polar residues. The active site of *Streptomyces griseus* aminopeptidase has been fully characterized [63]. It contains a unique binding site consisting of Glu, Asp and Arg residues, which play important roles in the binding and orientation of both substrate and the product of the catalytic reaction. Glu and Tyr are directly involved in the catalytic mechanism and play an important role in stabilization of tetrahedral transition state of the enzyme substrate complex. The analysis of bound and unbound states of fructose 1,6-bis(phosphate) aldolase show that the major structural differences are at 3 positions occupied by Lys, and 2 Arg residues [64]. Although none of these are catalytic residues, their mutations lead to differing effects on k(cat) and K(m). In the unbound state one of the Arg is involved in the formation of a salt-bridge, while in the bound state it interacts closely with the substrate. These residues play important roles in positioning the substrate at the binding site for catalysis [64]. The binding site analysis of several enzymes, including acetyl choline esterase, lysozyme and trypsin, show the optimized electrostatic complementarity between the binding site and the ligand (Honig and co-workers. URL: http://honiglab.cpmc.columbia.edu/). Theoretical studies have shown large electrostatic contributions to catalytic effects of enzymes [65-67]. A catalytic antibody has been shown to stabilize the transition states electrostatically through H-bonding and ionic interactions [60].

THE CASES

Barnase-Barstar Complex

An extra-cellular ribonuclease barnase (from *Bacillus amyloliquefaciens*) and its natural inhibitor barstar complex has been studied by several groups, using theoretical and experimental approaches. An exceptionally tight barnase-barstar binding ($K_d \sim 10^{-14}$) make this complex an ideal model to study the structural and thermodynamic determinants of high affinity, or to understand the fundamentals of molecular recognitions. The maximum diffusion-controlled rate constant for the collision of molecules in solution, calculated from Einstein-Smoluchowski equation, is 10^9 -10^{10} M^{-1} s^{-1}. For biomolecules the associations would be lesser by 3-4 orders of magnitude. However, barnase associates very rapidly with barstar (>5 X 10^9) [63]. The binding site of barnase and barstar has complementary positive and negative charges, respectively. Site-directed mutagenesis of charged residues and studies of the effect of electrostatic screening by salt have shown that the electrostatic forces enhance the association rate by 500-fold [68]. Both the long range electrostatic interactions followed by short range electrostatic interactions during docking were proposed to be the mechanism behind the high affinity binding. The complex has many salt-bridges and H-bonds at the binding interface [69]. The electrostatics thus play a major role in determining high affinity and tight binding. Robust electrostatic properties would make the binding site conformationally constrained and very specific. This is corroborated by the fact that barnase-barstar binding does not involve significant conformational changes [69,70]. The calculations of electrostatic free energy relative to hydrophobic isoster showed that barstar is electrostatically optimized for tight binding to barnase, where charged residues Asp 35, Asp 39 and Glu 76 contributed -4 - -12 kcal/mol towards the binding [71]. The computation of the binding charge desolvation and interaction potential also show high electrostatic complementarity at the binding site of barnase for barstar, or *vice versa* [71,72].

Cytochrome *c* Peroxidase-Cytochrome c Complex

The high affinity complex between electron transfer partners, cytochrome *c* peroxidase (296 residues) and cytochrome *c* (108 residues) has been extensively studied both experimentally and computationally. The binding site of cytochrome c peroxidase is negatively charged, while that of cytochrome *c* is positively charged. Molecular dynamics (MD) simulations have shown that they associate through electrostatic steering [73]. Brownian dynamics simulations show that experimentally measured high association rates depended strongly on the ionic strength of the solvent [57]. It was further shown to be correlated with the existence of strong electrostatic interactions, and high orientational electrostatic steering in the associations [57]. The binding affinity between them decreases with increase in ionic strength [74], consistent their electrostatic driven binding.

Proteinase-Proteinase Inhibitor Complex

Proteinase-proteinase inhibitor complex has been widely studied to understand the fundamentals of protein-protein interactions and molecular recognitions [75-78]. Their interfaces bury upto 1600 $Å^2$ of area. The electrostatic interactions drive protein-inhibitor binding [79], where H-bonds and salt-bridges contribute favorably for complexation. The complex formation has hydrophobic component and electrostatic component *via* H-bonds [13], with on average 9(+/-5) inter- molecular H-bonds. 12 non-homologous serine protease-inhibitor complexes were studied for the residue type involvement at the binding interface, and for main-chain side-chain energetic contributions towards binding [46]. The study highlights that protease-inhibitor mainly interacts *via* main-chain atoms [46], suggesting a closer and specific interactions, compared to antibody-protein antigen interactions. Antibody-protein antigen interactions mainly involve side-chain connections [27, 46], and the predominant contributions towards the binding is *via* side-chain interactions. The study on residue wise free energy contributions show that Arg and Lys contributed significantly towards the interaction enthalpy, 18 % and 9 %, respectively. It has also been shown that non-entropic residues contribute predominantly towards the binding in a proteinase-inhibitor complex [76].

Mesophilic and Thermophilic Glutamate Dehydrogenase

An increased number of charge-charge interactions, the networking of salt-bridges and long range electrostatic interactions has been proposed to account for the stability of

proteins from thermophilic sources. The X-ray crystal structures of mesophilic and thermophilic glutamate dehydrogenase (GD), from *Clostridium symbiosum* and *Pyrococcus furiosus*, respectively, are available. The structures from both the sources are highly similar [12]. The thermophilic GD is an extremely thermostable enzyme, with a half life of 12 hrs at 100°C, and melting temperature (T_m) of 113°C [80], in contrast to mesophilic GD, which has a T_m of 55°C. This provides us with an opportunity to study the structural and thermodynamic determinants of protein stability.

A correlation between salt-bridges, and their networks, with thermostabilities of glutamate dehydrogenase have been shown [81]. The thermophilic GD has ~70 % increase in the occurrence of salt-bridges for its whole hexameric functional unit [82]. Furthermore, the salt-bridges and their networks are significantly stabilizing towards binding in thermophilic GD, in contrast to their near neutral nature in mesophilic GD. Most of the additional salt-bridges present in thermophilic GD, where found around the active site [12], suggesting a relatively rigid binding site, resulting a higher energy barrier for unfolding or any conformational deformations at the elevated temperatures. GD from the thermophile *Thermococcus litoralis* is significantly less stable than GD from *P. furiosus*. Consistently, *T. litoralis* GD has fewer salt-bridges, modified H-bonding patterns, and lesser packing [83]. *P. furiosis* GD has the largest salt-bridge network, made up of 18 charged residues, at the dimer interface [39]. *P. furiosis* has significantly more favorable electrostatic contributions to folding, compared to mesophilic GD.

Lumazine Synthase

Lumazine synthase (LS) catalyses the penultimate step in riboflavin biosynthesis [84]. Lumazine synthase capsids from mesophilic *Bacillus subtilis* and thermophilic *Aquifex aeolicus* differ in their T_m by 27°C. LS from *B. subtilis* and *A. aeolicus* contains 60 homologous sub-units, arranged in icosahedral symmetry [85]. The RMSD between mesophilic and thermophilic LS is small (0.80 Å) [86]. The thermophilic enzyme has much higher number of surface charged residues, as well as a larger number of ion-pairs per subunit, compared to mesophilic LS. In thermophilic LS many salt-bridges are networked. A network of six salt-bridges is proposed to stabilize the inter-subunit interface. This salt-bridge network plays a functional role in maintaining the icosahedral structure [86]. The observation is consistent with the studies on structural comparisons of thermophilic and mesophilic proteins, showing an increased number of surface ion-pairs and the energetic optimization of ion-pair interactions in thermostable proteins [e.g. 8, 9, 87]. Thermophilic LS show significant differences in surface electrostatic properties, as compared to its mesophilic counter-part (Fig. **4**).

Antibody-Antigen Complex

Antibody-antigen complexes have long served as a model system to understand the fundamentals of protein-protein interactions and molecular recognition, both experimentally [88-93] and theoretically [94-96]. High affinity antibodies, very specific towards their antigens [43], or proposed to have "lock and key" type of binding [97], have higher electrostatic interactions with their antigens [94]. Antibody-protein antigen complexes, which bury over 1200 $Å^2$ surface area, derive about 70% of binding energy contributions from just six polar and charged residues- Tyr, Asp, Asn, Ser, Glu and Trp. Furthermore, about 50 % of the binding energy is contributed by four polar/charged residues-Arg, Lys Asn and Asp [46]. To date X-ray crystal structures of seven monoclonal anti-HEL antibodies complexed with HEL are available. Structural and thermodynamic analysis of these complexes have not only provided the basic information of binding, or molecular recognition "hot-spot" epitope and paratope residues but have also highlighted how large protein-protein complexes associate. Charged/polar epitope residues contribute the most towards free energy of binding in these antibody-HEL complexes. Lys at positions 96 and 97, Tyr at position 20, among thirteen epitope residues, contribute more than -4.0 kcal/mol towards the high affinity anti-HEL antibody- HH10-HEL binding [52]. Similarly in the anti-HEL antibody-HyHEL63, which recognizes the same epitope with similar affinity, Lys at position 97, among all the epitope residues, contributed the most (~ -3.6 kcal/mol), towards HEL binding [98]. Large scale computational analysis of HH10 family of antibodies show that Lys at position 97 not only forms an strong inter-molecular salt-bridge, with the electrostatic strengths ranging from -1.0 to -11.0 kcal/mol, but also participates in the formation of a very strong inter-molecular salt-bridge pentad (electrostatic strength: -34.0 kcal/mol) in a very specific and high affinity anti-HEL antibody- HyHEL26-HEL complex formation [27]. Isothermal titration calorimetry showed that in anti-HEL antibody HyHEL5 breaking an inter-molecular salt-bridge results in decreased affinity towards HEL by 40,000-fold, when Glu was mutated to Gln [50]. Furthermore, mutating a charged residue, involved in a salt-bridge, always yielded decreased binding affinities of HyHEL5 towards HEL [7]. These findings clearly show that charged residues play major roles in binding of high affinity monoclonal antibodies, *via* forming strong inter-molecular salt-bridge interactions. The long-range electrostatic effects have also been shown to enhance associations of high affinity antibodies, *via* electrostatic steering [99].

ELECTROSTATICS IN MEMBRANE PROTEINS

The lipid bilayer of membranes is hydrophilic on outer sides, and hydrophobic in between. The embedded transmembrane proteins are arranged in such a way that their hydrophilic region associates with the outer hydrophilic part and hydrophobic regions are in contact with inner hydrophobic part. Membrane proteins can consists a bundle of transmembrane α-helices, like 7-transmembrane helices in bacteriorhodopsin, or they can consists of several β-strands forming a channel, like in porin. The transmembrane α-helices of the photosynthetic reaction center are built up from continuous regions of predominantly hydrophobic residues.

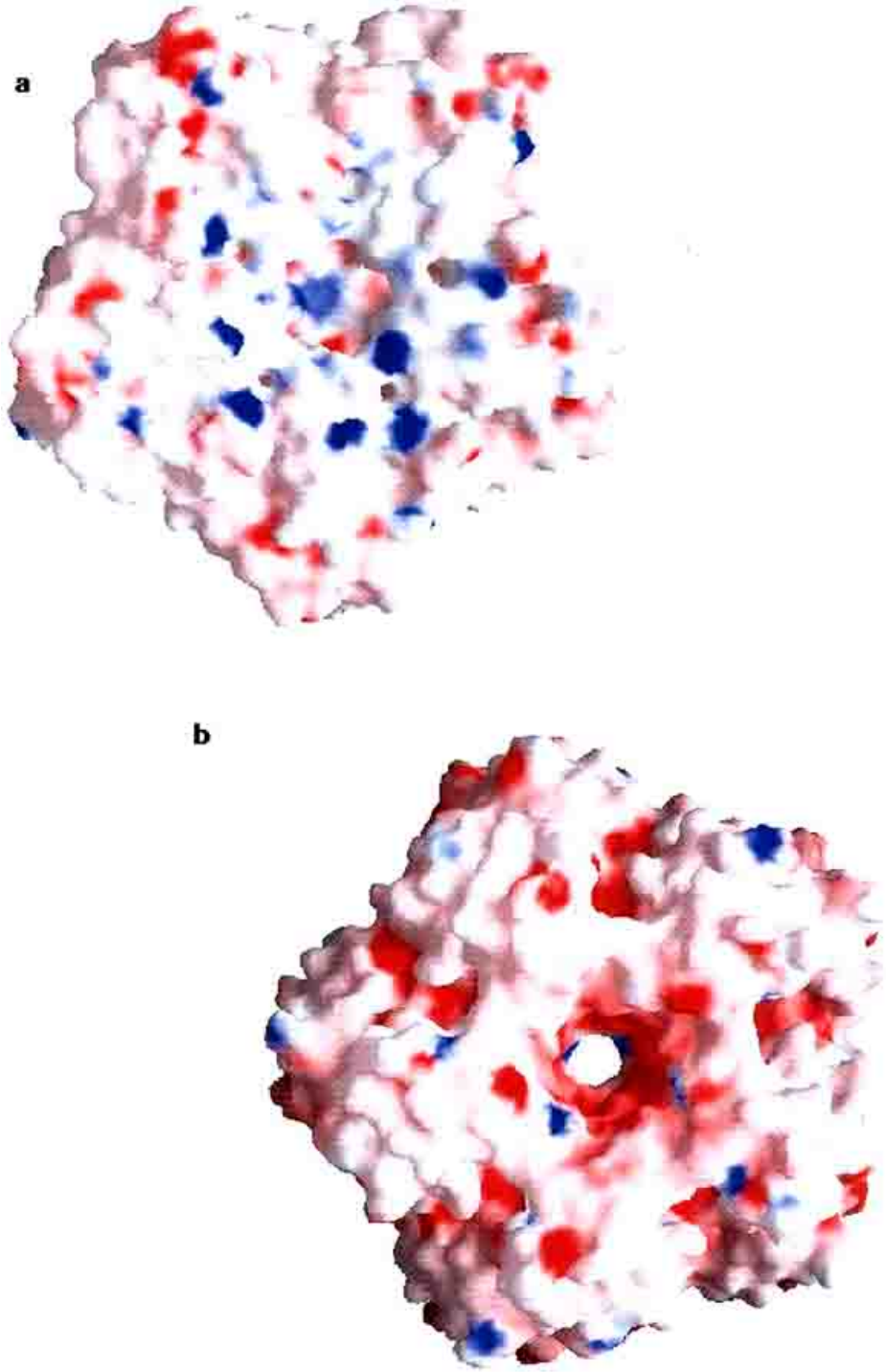

Fig. (4). The differences in surface electrostatic potentials between mesophilic (a: PDB id-1rvv, from *Bacillus subtilis*) and hyperthermophilic (b: PDB id-1hqk, from *Aquifex aeolicus*) Lumazine synthase. Figure is generated using GRASP [112], which solves Poisson Boltzmann equation and dispalys the electrostatic potential on the molecular surface.

The role of electrostatics in the stability and function of membrane proteins has not been thoroughly investigated. Charged residues present in the membrane spanning region have been shown to have functional importance. It has been suggested that long range electrostatics play a role in rapid kinetics and high specificity of electron transfer reaction in the photosynthetic reaction center of *Rhodobacter sphaeroides* [100]. However, another study suggest that charged side-chains contribute only marginally towards electrostatic energies in the reaction center [101]. A possible

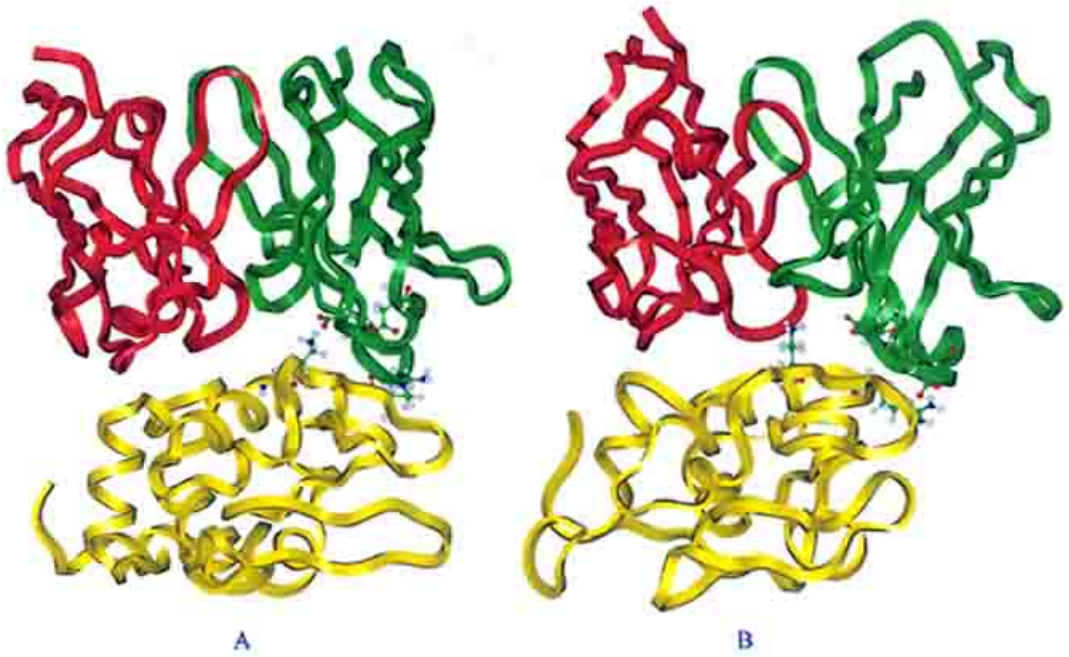

Fig. (5). X-ray crystal structure (A) and conformation at 1Nsec time step of 100°C MD simulation (B) of HyHEL63-HEL complex. Light chain, heavy chain and lysozyme are shown in red, green and yellow colors, respectively. Inter-molecular ion-pair interactions are shown by their side chains (see Figure 7 for details).

salt-bridge in lactose carrier protein play a structural role [102]. Charged residues Lys and Arg in membrane spanning region of prostaglandin transporter are critical for substrate translocation, where Lys likely contributes towards electrostatic binding of the anionic substrate [103]. Main-chain--main-chain inter-helical hydrogen-bonds in glycophorin A was suggested to stabilize helix-helix interactions [104]. The repellent effect of same charges, from aspartate and glutamate, between transmembrane helices of T-cell receptor plays a functional role [105]. The favorable interhelical interactions between Aspartate and Lysine was suggested important for TCR complex formation [106,107].

CHARGED AND POLAR INTERACTIONS DURING HIGH TEMPERATURE MD SIMULATION SIGNIFY THEIR STRUCTURAL / FUNCTIONAL SIGNIFICAN CE

HH63 is a monoclonal high affinity antibody, K_A=3.5 X 10^8, towards HEL. The X-ray crystal structure of the complex- HH63-HEL is available at 2.0 Å resolution (PDB id: 1dqj; [98]). 17 out of 21 epitope residues recognized by HH63 are either polar or charged, among which there are 7 charged residues. The inter-molecular interactions involve van der Waals contacts, hydrogen-bonds and a salt-bridge. Among 21 epitope residues there are two glycine residues. A short MD simulation revealed important inter-molecular salt-bridge and H-bonds in HH63-HEL (Sinha *et al.*, unpublished results) and HH10-HEL [108] associations.

We have performed high temperature 1NSec Molecular Dynamics (MD) simulation of HH63-HEL complex to ascertain "hot-spot" of molecular interactions. The conformation at 1NSec of 100°C MD simulation was inspected for the presence of any salt-bridge and inter-molecular H-bonds. The root mean square deviations (RMSD) between the starting X-ray crystal structure and the conformation at 1NSec was 3.3 Å, suggesting a significant difference between the two conformations (Fig. **5**). Fig. (**6**) shows that the HH63-HEL interface at 1NSec time step is not as compact as it is in the X-ray crystal structure. Table 1 shows salt-bridges and inter-molecular H-bonds in HH63-HEL complex at the 1NSec time step of 100°C MD simulation. Even at such high temperature the conformation contains one inter-molecular salt-bridge and nine inter-molecular H-bonds. The salt-bridge and H-bonds are of functional significance. They involve experimentally shown "hot-spot" epitope residues [98, 109, Li *et al.*, unpublished results). Asp at position 27 of the heavy chain maintains an inter-molecular salt-bridge with HEL (Table 1). 1NSec room temperature MD simulation has shown that Asp27 has close range interactions with eight HEL epitope residues (Sinha and Smith-Gill, unpublished results). The study clearly suggest that Asp27 is a "hot-spot" paratope residue for inter-molecular interactions. Fig. **7** shows inter-molecular ionic interactions at 1NSec time step of 100°C MD simulation. It is clear that although the HH63-HEL interface has altered significantly due to the high temperature, the "hot-spot" inter-molecular interactions are still maintained. This further highlights the structural/functional importance of charged/polar interactions at the binding site, or their governing role in binding of HH63-HEL, and likely in many protein-protein complexes.

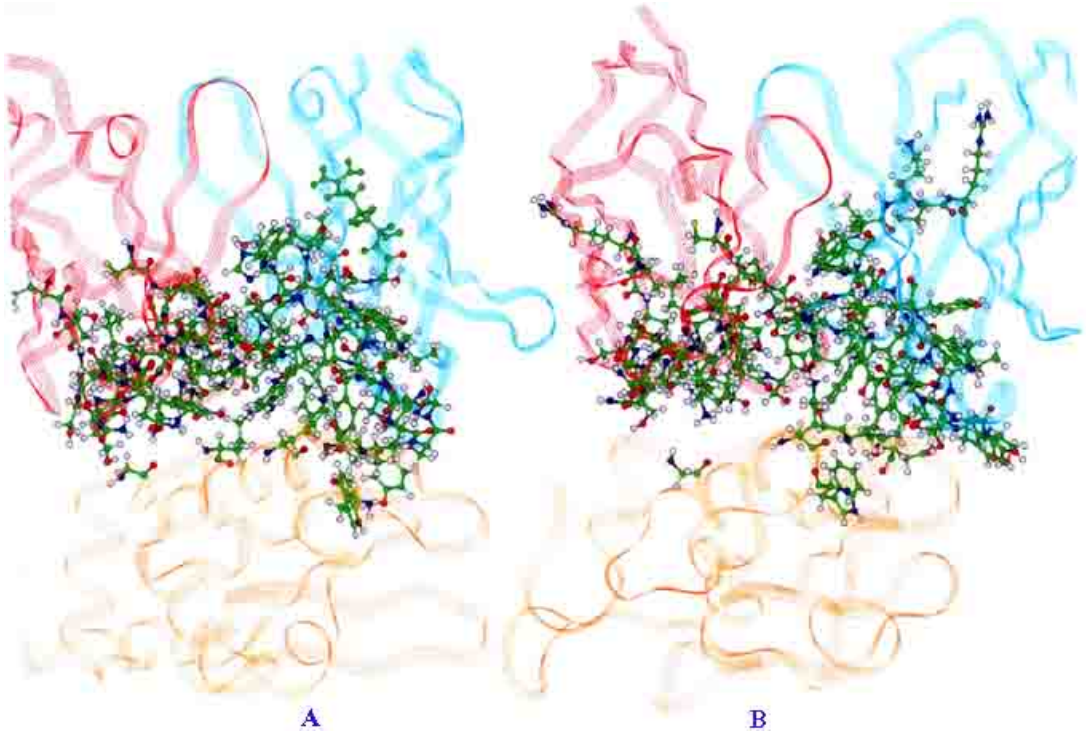

Fig. (6). X-ray crystal structure (A) and conformation at 1Nsec time step of 100°C MD simulation (B) of HyHEL63-HEL complex illustrating the interactions at the binding interface. Light chain, heavy chain and lysozyme is shown in red, cyan and brown color ribbons, respectively. All six CDRs are shown by residue side-chains, colored by atom types. The "hot-spot" epitope residues are also shown with their side-chains, colored by atom types.

Table 1. Salt-bridges and Inter-Molecular H-Bonds in a MD Conformer

Interaction	Corresponding region	Intra-molecular	Inter-molecular
Salt-bridges			
$Asp27_H$--$Arg73_Y$	CDR-H1--Epitope		✓
$Glu7_Y$--$Lys33_Y$	HEL	✓	
MC-MC Hydrogen-bonds			
MC-SC Hydrogen-bonds			
O $Ile29_L$-Nζ $Lys13_Y$	CDR-L1--Epitope		✓
O $Asn31_L$-Nζ $Lys96_Y$	CDR-L1--Epitope		✓
$Nδ^2$ $Asn92_L$-O $Asn19_Y$	CDR-L3--Epitope		✓
Oγ $Ser93_L$-N $Arg21_Y$	CDR-L3--Epitope		✓
Oγ $Ser52_H$-N $Asp101_Y$	CDR-H2--Epitope		✓
N $Tyr53_H$-$Oδ^2$ $Asp101_Y$	CDR-H2--Epitope		✓
Oγ $Ser56_H$-N $Gly102_Y$	CDR-H2--Epitope		✓
OH $Tyr58_H$-N $Gly102_Y$	CDR-H2--Epitope		✓
SC-SC Hydrogen-bonds			
OH $Tyr50_H$-NH2 $Arg21_Y$	CDR-H2--Epitope		✓

Salt-bridges and inter-molecular H-bonds in conformation at 1NSec time step of 100ºC MD simulation. Intra and inter-molecular interactions are marked. Three letter residue code is followed by position, which is followed by chain identification. Standard atom codes, and their positions, are listed in case of H-bonds. MC-MC: Main chain-main chain. MC-SC: Main chain-side chain. SC-SC: Side chain-side chain.

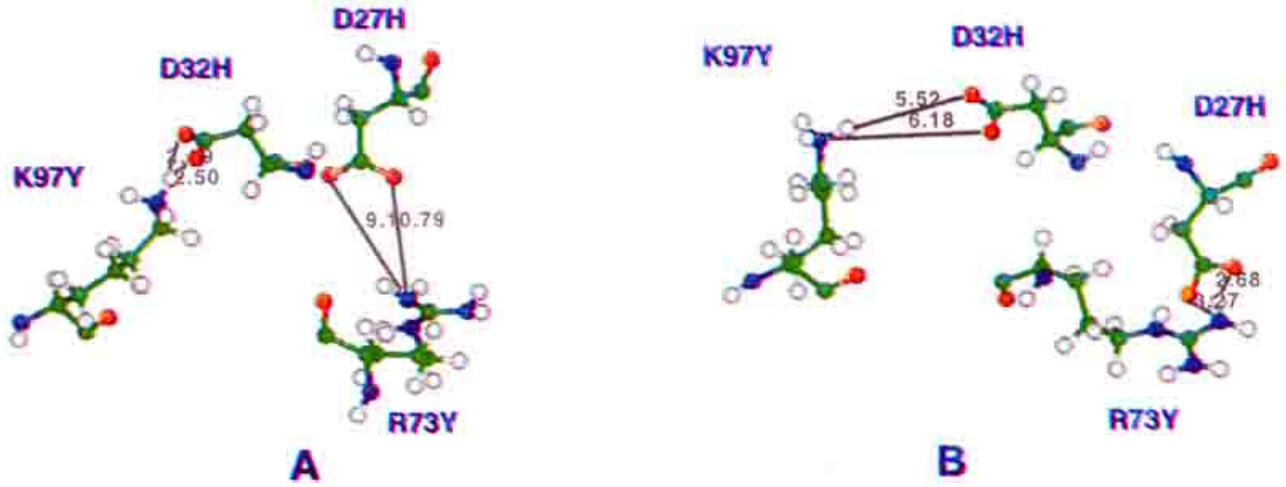

Fig. (7). Inter-molecular ion-pair interactions in X-ray crystal structure (A) and conformation at 1NSec 100°C MD simulation (B). The ion-pair forming side chains are displayed as they map in 3-D structure, colored by atom types. One letter residue code is followed by position, which is followed by the chain identifications.

BINDING MECHANISMS IN BARNASE-BARSTAR AND HYHEL63-HEL COMPLEXES

We have discussed that both barnase-barstar and HyHEL63-HEL complexes bind with high affinity. Tidor and co-workers have shown the electrostatic complementarity of barnase towards barstar is optimized [71,72]. The display of electrostatic potentials at their binding site also shows clear electrostatic complementarities between barnase and barstar [72]. However, displaying electrostatic potentials at HH63 and HEL binding sites do not show as clear electrostatic complementarities between them (Fig. **8**). Furthermore, the calculations of residual potentials (the sum of charge desolvation upon binding and interaction potential), according to the method devised by Lee and Tidor [71,72] also do not show significant electrostatic complementarities between HyHEL63 and HEL (not shown). Nonetheless, HH63-HEL has significant intra and inter-molecular H-bonds. Many of inter-molecular H-bonds involve "hot-spot" epitope residues, experimentally shown to contribute towards HH63-HEL binding. A short MD simulation has revealed the presence of an inter-molecular salt-bridge, $Asp32_H$[&]-$Lys97_Y$, which contributes -4.9 kcal/mol (Sinha *et al.*, unpublished results). Furthermore, many of the intra-molecular binding site salt-bridges are also shown to be structurally and functionally important in HH63-HEL binding (Sinha *et al.*, unpublished results). These salt-bridges involve "hot-spot" epitope and paratope residues; They are present in other antibody-HEL complexes, belonging to the same family; They recur during MD simulation. Surface plasmon resonance spectroscopy studies on Ala mutants of five charged or polar residues show that these residues contribute -1.2 to -3.6 kcal/mol towards HH63-HEL association [98]. $Lys97_Y$, which forms an inter-molecular salt-bridge contributes -3.6 kcal/mol towards the association [98]. All of these findings suggest the importance of short-range electrostatic interactions in HH63-HEL association. We have shown for HH10-HEL binding that the apparent "electrostatic steering", which manifests as a faster net association rate actually acts post-collision, where short range electrostatic interactions, H-bonds and ion-pairs, stabilize the encounter complex [27]. This is also consistant with the finding that electrostatically favored orientation gives a mismatch of about 180° [57]. In these antibody-antigen complexes electrostatics seem to play role post-collision by stabilizing the encounter complex, rather than enhancing diffusional encounter, as in associations of barnase-barstar and cytochrome-*c* peroxidase-cytochrome *c* [57]. The mechanisms may be different in barnase-barstar and HH63-HEL associations, it is electrostatically assisted in both the cases. Electrostatics play key roles in the high affinity binding of both the complexes, although at different steps of the association process.

CONCLUSIONS

The folding of a nascent polypeptide chain is hydrophobically driven. This is mostly true for single domain small proteins. The non-bonded polar/charge interactions, such as H-bonds and salt-bridges, mainly define secondary and tertiary structure formation. The characteristics of distinctive 3-D fold are determined by the electrostatic properties, so as the specificities of active and binding sites. Stabilizing as well as destabilizing salt-bridges occur in proteins. Both play roles in binding and function. A destabilizing or a marginally stabilizing salt-bridge would allow conformational flexibility or a specific conformation [23], while an stabilizing salt-bridge may rigidify local structure, disallowing conformational fluctuations [20, 24]. Electrostatic forces govern structural/functional properties of proteins in two ways; i) locally- by short range electrostatic interactions, by forming H-bonds and ion-pairs; ii) globally-

[&] Subscript is chain identification. H: Heavy chain, Y: Lysozyme

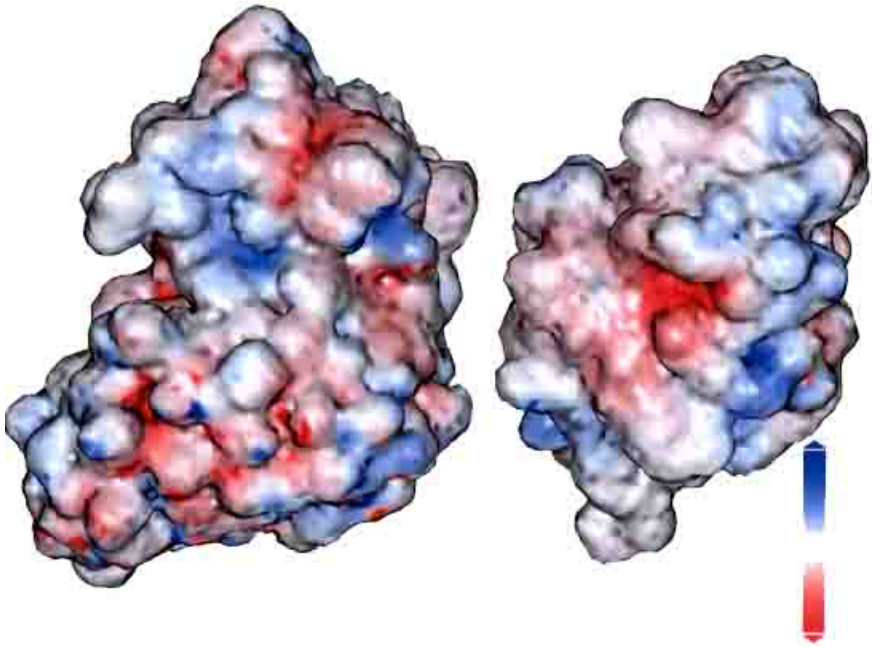

Fig. (8). Electrostatic potentials, calculated using DELPHI [113], are displayed on unbound HH63 (i) and HEL (ii) binding sites, using InsightII (ACCELRYS Inc.).

by influencing the over-all electrostatic environment of the protein. For instance, distribution of charge residues near to a salt-bridge, or their networks, may facilitate the favorable electrostatic interactions between salt-bridging side-chains. This property has been described as the protein term, which addresses the electrostatic interactions between charges in protein and charges in salt-bridging side-chains [12, 23, 24]. The over-all charge distribution at the binding, or surrounding active site, plays a major role in providing the complementarities between binding partners. The charge complementarities between protein-protein binding partners enhance diffusional encounters [54-56]. Intra and inter-molecular salt-bridges, and their networks, limit flexibility [24, 27, 31]. The short range electrostatic interactions are avoided in flexible regions of proteins [24, 27, 31]. Thus, local electrostatic interactions define flexible and rigid regions of proteins. Inherent flexible regions are important for protein function [24-26, 108]. For example: Coil movements around the enzyme active site; domain movements in adenylate kinase and calmodulin; conformational changes during "induced fit" type of binding; domain swapping during protein oligomerization. Electrostatics is crucial for defining specificity of folding, binding and function. While hydrophobicity is associated with conformational flexibility, and allows protein movements [24, 31], where the extent of hydrophobicity regulates the extent of movements [24]. On qualitative comparisons of three antibody-antigen complexes, belonging to the same family, the differeneces in their hydrophobic contributions towards antigen binding were small. Although significant differences were found in the electrostatic contribtions, among the complexes. The most specific, and least cross-reactive binding was most electrostatic in nature [27]. This may imply that during the maturation of a very specific binding site the electrostatic features are enhanced, while hydrophobicity is optimized to begin with.

METHODS

Molecular Dynamics Simulation

1NSec MD simulations were performed at constant temperature and volume, in NVT canonical ensemble, in the cubic periodic boundary conditions, using C-DISCOVER at the INSIGHT-II interface. The system consisted of variable domains, lysozyme, water molecules in the crystal structure and another 7286 water molecules, making total of 27,105 atoms perunit cell. The system was subjected for equilibration for 10 Ps, before collecting data. All the atoms in the system were considered explicitly, and the interactions were computed using CFF91 force field [110]. Total energy, potential energy, kinetic energy and temperature showed steady behavior over the production run, suggesting that sound equilibration was attained during the data collection stages.

Salt-Bridges and H-Bonds

The definition of a salt bridge is taken from Kumar and Nussinov [111]. According to this definition two oppositely charged residues are inferred to form a salt bridge if they satisfy the following two criteria:

(i) Centroids of side-chain functional groups in oppositely charged residues lie within 4.0 Å of each other.

(ii) At least a pair of Asp or Glu side-chain carboxyl oxygen and Arg, Lys and His side-chains nitrogen atoms are within a 4.0 Å distance.

The presence of a hydrogen bond is inferred when two non-hydrogen atoms with opposite partial charges are within a distance of 3.5 Å.

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ABBREVIATIONS

CDR	=	Complementarity determining region
HEL	=	Hen egg white lysozyme
HH antibody	=	HyHEL antibody
MD	=	Molecular dynamics
GD	=	Glutamate dehydrogenase
LS	=	Lumazine synthase

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