Protein flexibility and electrostatic interactions

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In this paper we address the interrelationship between electrostatic interactions and protein flexibility. Protein flexibility may imply small conformational changes due to the movement of backbone and of side-chain atoms, and/or large-scale molecular motions, in which parts of the protein move as rigid bodies with respect to one another. In particular, we focus on oppositely charged side chains interacting to form salt bridges. The paper has two parts: In the first, we illustrate that the majority of the salt bridges are formed within the independently folding, compact hydrophobic units (HFUs) of the proteins. On the other hand, salt bridges forming across the HFUs, where one amino acid resides in one HFU and its pairing "spouse" in a second, appear to be avoided. In the second part of the paper, we address electrostatic interactions in conformational isomers around the native state. We pick the protein Cyanovirin-N as an example. We show that salt bridges and ion pairs, with less optimal geometry, often interconvert between being stabilizing and destabilizing. We conclude that the stabilizing, or destabilizing, contribution of a salt bridge to protein structure is conformer-dependent.

Introduction

Salt bridges play important roles in protein structure and function, e.g., in oligomerization, molecular recognition, allosteric regulation, domain motions, flexibility, thermostability, and α-helix capping [1–7]. The electrostatic contribution to the free-energy change upon salt-bridge formation varies significantly, from being stabilizing to marginal to being destabilizing [8–17]. Disruption of the Glu34–Lys38 salt bridge in a DNA binding protein, HU from *Bacillus stearothermophilus*, reduces its thermal stability. On the other hand, the introduction of this salt bridge into a homologous HU from the mesophile *Bacillus subtilis* increases the stability of the protein [18]. Salt bridges are more frequent in proteins from thermophiles (i.e., organisms living in high temperatures) as compared to those from mesophiles (living under physiological conditions [7(b), 19]).

This paper discusses the interplay between protein flexibility and electrostatic interactions, particularly of salt bridges and ion pairs. Understanding protein flexibility is important for protein function and for rational drug design [20]. Protein flexibility can imply two distinct phenomena. The first is large-scale molecular motions [21], in which two (or more) parts of a protein move as rigid bodies with respect to one another. This type of motion is often observed in molecular events such as substrate/ligand binding or allostery [22]. Such motions

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can usually be detected from a comparison of crystal structures of a protein in the bound and unbound states [23, 24]. The results of such comparisons have been classified and cataloged in a database of macromolecular movements [21]. The second type involves small-scale protein motions, reflected in an ensemble of conformational isomers of a protein around its native state at the bottom of its energy funnel [25]. This type of motion can be studied in atomic detail by molecular dynamics simulations or by analyzing the nuclear magnetic resonance (NMR) conformer ensemble for the protein.

The electrostatic interaction energies between a pair of charged residues are directly correlated with the pK_a values[1] of the individual charged residues [4]. The determination of pK_a shifts helps in understanding the enzyme catalytic mechanism. The formation/breakage of salt bridges can also affect protein stability. Recently, protein folding has been described in terms of multiple pathways gliding down the slopes of the funnel, with one (or a few) more prominent than the others. Kumar et al. [26] have proposed that energy landscapes are dynamic, changing with the protein environment, e.g., with pH, temperature, the presence/absence of ligands and of cofactors. The dynamic energy landscape reflects the redistribution of protein conformer populations in response to the environmental change. Electrostatic interactions affect and are affected by protein flexibility. Flexibility implies movement of atoms, residues, and fragments of the protein with respect to one another. These involve changes in both hydrophobic and electrostatic interactions. Changes in electrostatic interactions, such as the formation or breakage of salt bridges, implies changes in the pK_a of the charged-residue side chains and in protein stability. Hence, it is important to understand the interplay between protein flexibility and electrostatic interactions.

Here, we compare salt bridges and their stabilities in protein structures determined by X-ray crystallography and/or by NMR. X-ray crystallography and NMR are two widely used techniques that provide protein structural information at atomic resolution. Recently, we have analyzed a database of 222 nonequivalent salt bridges from 36 nonhomologous proteins whose high-resolution (1.6 Å or better) crystal structures are available in the Protein Data Bank (PDB) [17, 27]. We observed that most of the salt bridges have stabilizing electrostatic contributions to the proteins and are formed at close *sequence* separations. In the first part of the paper we show that most of these salt bridges are formed within the hydrophobic folding units (HFUs) of these proteins. We further compare the electrostatic strengths of intra-HFU and inter-HFU salt bridges, and show that intra- and inter-HFU salt bridges have similar stabilities. However, substantially fewer inter-HFU salt bridges are formed than intra-HFU ones.

In the second part of the paper, we present the fluctuations in electrostatic strength of a salt bridge in the NMR conformer ensemble of Cyanovirin-N, a potent HIV-inactivating protein. In the crystalline state, Cyanovirin-N forms a domain-swapped dimer [28]; however, it is a monomer in solution [29]. Our calculations illustrate that electrostatic interactions are sensitive to protein flexibility. The stabilizing (or destabilizing) contribution of a salt bridge depends on its location and the geometrical orientations of the charged-residue side chains with respect to one another, and with respect to their environment in the protein structure. Hence, its contribution is conformer-population-dependent.

Taken together, these results indicate that for the larger-scale, slower modes of motion, such as those of hinge-bending, electrostatic interactions are largely avoided. The electrostatic interactions may rigidify the moving parts of the protein and, hence, hinder its biological activity. A hallmark of biological function is the ability of domains and of substructural units to move with respect to one another. A typical example is that of active sites, which frequently reside at the interdomain interface. On the other hand, when considering the smaller-scale motions around the native state, an ion pair cannot be uniquely described as stabilizing or destabilizing to the protein structure. Its contribution fluctuates in solution, and is dependent on which conformer is examined. Protein flexibility is a composite of mixed modes of motion. Within the scope of this issue of the *IBM Journal of Research and Development* and the insight which may be provided by computational studies, the goal of this presentation is to illuminate some aspects of the complex and intricate interplay of charge–charge interactions and the larger- and smaller-scale modes of protein motions.

Below, we detail the more technical aspects of our calculations.

[1] For weak acids, such as amino acid ionizable groups in proteins, the ionization equilibrium is given by

$$HA = H^+ + A^-.$$

The apparent equilibrium constant K_a for this ionization is

$$K_a = \frac{[H^+]\,[A^-]}{[HA]}.$$

Hence, the pK_a for the acid is given by

$$pK_a = -\log K_a.$$

The pK_a of an acid is also the pH ($= -\log [H^+]$) at which it is half dissociated. Each amino acid has at least two ionizable groups—an α-carboxyl group and a protonated α-amino group. Besides these, some amino acid side chains, such as those in charged amino acids, also contain ionizable groups. The pK_a values for the ionizable groups in amino acid side chains range from 3.9 (aspartic acid) to 12.5 (arginine). During enzyme catalysis, the apparent pK_a values for the side-chain ionizable groups in the enzyme active site amino acid residues shift upon ligand/substrate or cation/anion binding.

Salt bridges and computation of their electrostatic strengths

The method used to identify salt bridges has previously been described [17]. Two charged residues in a protein are inferred to form salt bridges if 1) their side-chain charged group centroids are within a 4-Å distance and 2) at least one pair of side-chain nitrogen and oxygen atoms in the two residues are within a 4-Å distance. Since most crystal structures do not contain coordinates of hydrogen atoms, only the nonhydrogen (heavy) atom coordinates are used to compute side-chain charged group centroids. The locations of charged residues in the protein are estimated by their accessible surface area (ASA) [30, 31] obtained by rolling a solvent (water) probe ball of radius 1.4 Å over the protein surface. Hence, a small ASA value for a charged residue indicates that it is mostly buried in the protein core. On the other hand, a large ASA value indicates that it lies on the protein surface. The hydrophobic folding units (HFUs) in the 36 nonhomologous proteins are identified using the method of Tsai and Nussinov [30].

Electrostatic strengths of salt bridges in protein crystal structures are calculated using the continuum electrostatic methodology [7(b), 16, 17]. The electrostatic contribution to the free-energy change upon salt-bridge formation is calculated relative to computer mutations of the salt-bridging-residue side chains to their hydrophobic isosteres. The hydrophobic isosteres are the salt-bridging-residue side chains with their partial atomic charges set to zero.

The total electrostatic contribution to the free-energy change upon formation of a salt bridge, $\Delta\Delta G_{tot}$, is the sum of three components:

$$\Delta\Delta G_{tot} = \Delta\Delta G_{dslv} + \Delta\Delta G_{brd} + \Delta\Delta G_{prt} .$$

The term $\Delta\Delta G_{dslv}$ is the sum of the unfavorable desolvation penalties incurred by the individual salt-bridging residues due to the change in their environment from a high-dielectric solvent (water) in the unfolded state to the low-dielectric protein interior in the folded state of the protein. The term $\Delta\Delta G_{brd}$ is the favorable bridge energy due to the electrostatic interaction of the side-chain charged groups with one another in the folded state of the protein. The term $\Delta\Delta G_{prt}$ is the electrostatic interaction of the salt-bridging side chains with the charges in the rest of the protein in its folded state.

An additional free-energy term called association energy, $\Delta\Delta G_{assoc}$, represents the desolvation of the salt bridge and the electrostatic interaction between the salt-bridging side chains, but does not consider the electrostatic interaction of the salt bridge with the rest of the protein. Hence, it represents the electrostatic contribution to the free-energy change upon salt-bridge formation in the absence of charges in the rest of the protein [16]. All of the energy values are presented in kcal/mol, and all of the calculations are performed using the DELPHI package [32–36]. This method was used extensively earlier [6, 7(b), 10, 16, 17, and Kumar and Nussinov, unpublished work], and experimental support for it has been reported [37, 38].

For the computational studies addressing large-scale motions, we utilize a database of 222 nonequivalent salt bridges from a set of 36 monomeric proteins. The composition of the database has been described in [17]. Large-scale flexibility can often be traced to a small segment of a protein. Below, we refer to it as *segmental flexibility*. Segmental flexibility can frequently be inferred through crystallizing proteins in different states, e.g., in the presence/absence of substrate(s), ligand(s), or cofactor(s), or other molecules. The movements in different protein parts due to ligand/substrate/cofactor binding are observed by comparing protein structures in the "open" and "closed" conformations, i.e., the bound and unbound forms. Such movements have been assembled and classified in the Database of Macromolecular Motions [21]. There are also computational approaches which analytically infer the location of the more flexible regions directly from the crystal structures [24, 39]. Large-scale motions most frequently involve hinge-bending. In hinge-bending motions, two parts of a protein (e.g., two fragments of the polypeptide chain, two domains, or two subunits) move with respect to one another in a direction which is perpendicular to the plane defined by the interface between the two parts [21]. When the motion of the parts is parallel to the plane of the interface, the motion is classified as shear.

Here we chose Cyanovirin-N as a model system for the study of fluctuations in electrostatic interactions in proteins around the native state. Since this kind of protein flexibility relates to the whole structure, we refer to it below as *systemic flexibility*. Both crystal and NMR structures are available for this potent HIV-inactivating protein [28, 29]. The crystal structure for the protein has a resolution of 1.5 Å [28]. The solution structure of Cyanovirin-N is also well-defined, since it was solved using 2509 NMR restraints [29]. The resulting conformer ensemble consists of 40 conformers. An average energy-minimized structure computed from the NMR conformer ensemble is also available. The protein data bank (PDB) entry codes are 2ezn (NMR conformer ensemble), 2ezm (average energy-minimized structure), and 3ezm (crystal structure). Cyanovirin-N contains 101 amino acids. The strengths of the electrostatic interactions for the ion pair E68–K84 in the crystal structure, the NMR average energy-minimized structure, and the NMR conformer ensemble were calculated in a manner similar to that

Table 1 Intra- and inter-hydrophobic folding unit (HFU) salt bridges in 36 nonhomologous monomeric proteins.

Protein PDB file	*No. of HFUs*	*No. of salt bridges*	*Intra-HFU salt bridges*	*Inter-HFU salt bridges*	*HFU–UA* salt bridges*	*UA*–UA* salt bridges*
1351	1	1	1	0	0	0
1531	2	7	5	1	0	1
1ads	2	15	13	2	0	0
1aky	3	5	3	1	1	0
1akz	1	6	3	0	1	2
1amm	2	3	3	0	0	0
1aop	3	16	14	1	1	0
1arb	1	7	7	0	0	0
1aru	2	7	5	2	0	0
1bfg	1	4	4	0	0	0
1bvd	1	6	6	0	0	0
1cex	1	3	3	0	0	0
1cyo	1	2	2	0	0	0
1dim	3	9	7	2	0	0
1edg	3	7	6	1	0	0
1fmk	4	9	6	1	2	0
1hmr	1	7	7	0	0	0
1igd	1	3	3	0	0	0
1mla	2	5	5	0	0	0
1orc	1	2	2	0	0	0
1phc	2	13	13	0	0	0
1ptx	1	2	2	0	0	0
1rcf	1	4	4	0	0	0
1rie	1	2	2	0	0	0
1rro	1	3	3	0	0	0
1ruv	1	3	3	0	0	0
1smd	4	16	12	4	0	0
1tca	2	2	2	0	0	0
1yge	3	23	8	0	3	12
1yna	1	1	1	0	0	0
2ayh	1	2	2	0	0	0
2dri	2	8	8	0	0	0
2end	1	5	5	0	0	0
2eng	1	5	5	0	0	0
2phy	1	3	3	0	0	0
3pte	2	6	5	1	0	0
Total	61	222	183	16	8	15

*UA stands for unassigned region of the protein—the region of the protein which could not be assigned to any hydrophobic folding unit. Hence, HFU–UA indicates a salt bridge with one residue falling in a hydrophobic folding unit and the other in an unassigned region of the protein. Both residues in a UA–UA salt bridge lie in the unassigned region(s) of the protein. Proteins are denoted by their PDB codes, and proteins containing more than one HFU are indicated in boldface.

described above for the 222 salt bridges in 36 nonhomologous protein crystal structures.[2]

Salt bridges and segmental flexibility

The 36 nonhomologous monomeric proteins [17] are cut into their hydrophobic folding units (HFU) [30]. Hydrophobic folding units are compact, stable substructures with a strong hydrophobic core, which preserve their structures in solution [30]. These substructures can swivel as rigid bodies with respect to one another, in a hinge-bending type of motion. Here we compare salt bridges formed within and across hydrophobic folding units (HFUs).

Of the 222 salt bridges, 183 (82.4%) are formed within the HFUs, and 16 (7.2%) across the HFUs. The remaining 23 (10.4%) salt bridges contain at least one residue which falls in a region that is not assigned to any HFU. **Table 1** lists the intra- and inter-HFU salt bridges in the 36 nonhomologous proteins. The proteins that contain more than one hydrophobic folding unit are indicated in boldface. Sixteen proteins in our database contain more than one hydrophobic folding unit. The total number of salt bridges in these 16 proteins is 152; of these, 115 (75.7%) are formed within HFUs (intra-HFU salt bridges)

[2] We use the standard letter codes for amino acids: E is glutamic acid; D is aspartic acid; K is lysine; and R is arginine. A notation such as E68–K84 means that glutamic acid numbered 68 in the sequence forms a salt bridge or an ion pair with lysine 84. A salt bridge is formed when the distance between charged groups is ≤4 Å. If the distance is greater than this value, the charge–charge interaction is denoted as an ion pair.

Table 2 Average values for the energy terms in various salt-bridge categories.

Salt-bridge class	$\Delta\Delta G_{dslv}$ (kcal/mol)	$\Delta\Delta G_{brd}$ (kcal/mol)	$\Delta\Delta G_{prt}$ (kcal/mol)	$\Delta\Delta G_{tot}$ (kcal/mol)	$\Delta\Delta G_{assoc}$ (kcal/mol)
All	$+6.54 \pm 5.48$	-6.34 ± 4.38	-3.86 ± 4.35	-3.66 ± 3.86	-3.64 ± 2.63
Intra-HFU	$+6.01 \pm 5.12$	-5.96 ± 4.20	-3.56 ± 4.09	-3.51 ± 3.79	-3.53 ± 2.64
Inter-HFU	$+10.82 \pm 6.24$	-8.99 ± 4.60	-6.93 ± 6.53	-5.10 ± 4.84	-4.42 ± 2.54

All: Whole dataset of 222 salt bridges [17]; intra-HFU: 183 intra-HFU salt bridges; inter-HFU: 16 inter-HFU salt bridges.

and 16 (10.5%) across the HFUs. Twenty-one salt bridges fall in unassigned regions. Of the 16 proteins that contain more than one HFU, six proteins do not contain any inter-HFU salt bridge. These observations indicate that most of the salt bridges are formed within the hydrophobic folding units rather than across them.

In the whole database of 222 salt bridges, 66 (29.7%) are buried in the proteins, with the average ASA (accessible surface area) for these salt bridges being less than 20%. Similarly, 44 (24.0%) of the 183 intra-HFU salt bridges are buried in the protein. On the basis of the overall distribution of buried and surface-exposed salt bridges in the database of 222 salt bridges, 54 ($66 \times 183/222$) intra-HFU salt bridges are expected to be buried. Hence, a smaller number of intra-HFU salt bridges are buried. However, this change in the proportion of buried salt bridges is insignificant at a 95% level of confidence, as determined by the change-of-proportion test [40]. On the other hand, 10 (62.5%) of the 16 inter-HFU salt bridges are buried. On the basis of the overall distribution of buried and surface-exposed salt bridges in the database of 222 salt bridges, only five ($66 \times 16/222$) inter-HFU salt bridges are expected to be buried. This increase in the proportion of buried inter-HFU salt bridges is significant at a 95% level of confidence. Because inter-HFU salt bridges lie at or near the interface between the two hydrophobic folding units, they are more likely to be buried in the protein.

Of 183 intra-HFU salt bridges, 153 (83.6%) have stabilizing electrostatic contributions; the remaining 30 (16.4%) are destabilizing to the protein structures that contain them. These observations agree well with those for all of the 222 salt bridges in the database. Of 222 salt bridges in our dataset, 190 (85.6%) are stabilizing and 32 (14.4%) are destabilizing [17]. On the other hand, all of the 16 inter-HFU salt bridges are stabilizing. On the basis of the distribution of stabilizing and destabilizing salt bridges in the dataset of 222 salt bridges, 14 of the 16 inter-HFU salt bridges are expected to be stabilizing, and the remaining two are expected to be destabilizing. Since the number of inter-HFU salt bridges is small in our database, we cannot extrapolate from our results that all inter-HFU salt bridges will be stabilizing. However, it is likely that a greater proportion of inter-HFU salt bridges are stabilizing than intra-HFU salt bridges. **Table 2** presents the average values of various energy terms for the 222 salt bridges, 183 intra-HFU salt bridges, and 16 inter-HFU salt bridges. Large standard deviations about the average values of various terms indicate large scatter in the data. However, it can be seen that intra-HFU salt bridges have an average electrostatic contribution that is similar to the average over the 222 salt bridges. On the other hand, inter-HFU salt bridges pay greater desolvation penalties, $\Delta\Delta G_{dslv}$, owing to their larger proportion of burial. Nevertheless, these larger desolvation penalties are overcome by stronger bridge ($\Delta\Delta G_{brd}$) and protein ($\Delta\Delta G_{prt}$) energy terms for the inter-HFU salt bridges. Since most of the intra-HFU salt bridges are buried in the protein core, electrostatic interactions between the charged-residue side chains constituting the salt bridges and the electrostatic interactions between the salt bridges and the neighboring charges suffer less solvent screening. Reduced solvent screening of the buried salt bridges is thought to be responsible for their greater strength in comparison with the surface-exposed salt bridges [17]. Similar principles may also apply for the inter-HFU salt bridges. A comparison of the relative magnitudes of the bridge and protein energy terms indicates that the protein energy term ($\Delta\Delta G_{prt}$) contributes to a greater extent toward the stability of inter-HFU salt bridges. From Table 2, it appears that inter-HFU salt bridges have greater average stability ($\Delta\Delta G_{tot} = -5.10 \pm 4.84$ kcal/mol) than intra-HFU salt bridges ($\Delta\Delta G_{tot} = -3.51 \pm 3.79$ kcal/mol). However, this difference is largely due to the inter-HFU salt bridge E27–R387 in 1smd (α-amylase). This salt bridge has very high stability (data not shown); in fact, this is the most stable salt bridge in our database [17]. If we remove this salt bridge from our list of inter-HFU salt bridges, the average $\Delta\Delta G_{tot}$ for the remaining 15 inter-HFU salt bridges is -3.94 ± 1.48 kcal/mol, indicating that intra- and inter-HFU salt bridges have similar stabilities.

On the basis of the results presented above, we can say that most of the salt bridges are formed within the hydrophobic folding units rather than across them. In terms of final electrostatic strengths, the intra- and inter-HFU salt bridges do not show significant differences.

Since hydrophobic folding units have been identified computationally, it is not known whether they actually move with respect to one another. Of the 16 monomeric proteins that contain more than one HFU, five have both bound and unbound structures and have been classified in the Database of Molecular Motions [21] as having hinge-bending swiveling. These are the proteins which in the PDB have been assigned entries 1ads, 1fmk, 1phc, 1tca, and 2dri. Three of these (1phc, 1tca, and 2dri) contain no inter-HFU salt bridge; 1ads contains two inter-HFU salt bridges, and 1fmk contains one inter-HFU salt bridge. The stabilities of these three salt bridges are similar to the average salt-bridge stability in our database.

In a related study, we have taken those examples from the Database of Molecular Motions [21] in which protein crystal structures are available both in the "open" and "closed" forms (Sinha, Kumar, and Nussinov, unpublished results); hence, the movements in these proteins are real. This comprehensive study analyzes electrostatic and hydrophobic interactions in building blocks [41], hydrophobic folding units [30], domains, and subunits of 25 proteins and compares inter-building-block/HFU/domain/subunit interactions with intra-building-block/HFU/domain/subunit interactions. While nonspecific hydrophobic interactions can be very substantial between the two parts (e.g., building blocks, HFUs, domains, or subunits) of a protein molecule, the number of hydrogen bonds and salt bridges which are formed is quite small.

It is reasonable to expect that hydrophobic and electrostatic interactions between two parts of a protein would be smaller than those within the two parts because the surfaces buried in interfaces between protein parts are smaller than the surfaces of the two parts themselves. In principle, if salt bridges were to be engineered in a protein in a random manner, a greater fraction of such salt bridges could be expected to fall within rather than across the domains. Similar experiments may be conceived for other types of interactions in proteins. However, these experiments would not explain the observed differences among the extents of hydrophobic and electrostatic interactions. In a related study by our group (Sinha, Kumar, and Nussinov, unpublished results), we have observed that the formation of inter-HFU salt bridges is avoided. The nonpolar-surface areas buried in the interfaces are typically one third of the nonpolar-surface areas buried within the parts. The ratio of intra- to inter-HFU salt bridges in the 16 proteins that contain more than one HFU is 115:16—approximately 7:1 in favor of the intra-HFU salt bridges. The significance of the decrease in proportion of inter-HFU salt bridges can be tested as follows. Let us assume that the distribution of inter- and intra-HFU salt bridges is similar to that of nonpolar-surface areas buried across and within the HFUs in the 16 proteins that contain more than one HFU. Hence, we would expect the number of inter-HFU salt bridges to be one third of the intra-HFU salt bridges. If N is the number of intra-HFU salt bridges, the sum of intra- and inter-HFU salt bridges will be $4N/3$. Since the sum of intra- and inter-HFU salt bridges in the 16 proteins is 131 (115 intra- and 16 inter-HFU salt bridges), the expected numbers of intra- and inter-HFU salt bridges are 98 and 33, respectively. Hence, the actual number of inter-HFU salt bridges (16) is less than half of the expected number (33). This decrease in the observed number of inter-HFU salt bridges is significant at a 95% confidence level as determined by the change-in-proportion test [40]. The observed lower occurrence of salt bridges between the two parts of a protein molecule than within the parts indicates that such interactions are avoided at the interfaces. These observations can be rationalized: Electrostatic interactions such as salt bridges and hydrogen bonds are specific, and their formation can constrain the movement of the molecular parts with respect to one another. Breaking such electrostatic interactions may involve overcoming larger energy barriers [38]. On the other hand, hydrophobic interactions may be pictorially viewed as a nonspecific glue [42].

Salt bridges and systemic flexibility

Systemic protein flexibility around the native state arises because of small conformational fluctuations in the protein backbone and side-chain atoms. Protein crystal structures typically yield static information on the protein structure. Small-scale motions due to systemic flexibility can be deduced from atomic thermal parameters (B-factors) in the protein crystal structures. However, B-factors also depend upon electron density smear, and hence upon the resolution of the structure [43]. There are two alternate ways in which one can study systemic protein flexibility: molecular dynamics simulations and nuclear magnetic resonance (NMR). Both techniques have limitations. Currently, most molecular dynamic simulations are limited to relatively small time scales (of the order of 1 ns), even though a 1-μs-scale simulation has recently been reported [44]. Multiple long simulations are needed to obtain a reasonably broad conformational sampling around the protein native state [45]. On the other hand, a typical protein NMR experiment yields an ensemble of conformers satisfying a list of nOe (nuclear Overhauser effect) distance restraints among hydrogen atoms close in space (1.8 Å to 5.0 Å [46]). Dynamic information on the proteins is implicit both in these restraints and in regions where they are absent. However, there are two shortcomings: the conformational space covered by the NMR ensemble and the quality of the conformers. These shortcomings arise both because of insufficient data and because of the structure calculation protocol. Further, it is

often difficult to distinguish the true dynamic behavior of the protein from artifacts produced by these shortcomings [47]. Nevertheless, in the literature there have been a number of reports which indicate that the conformational sampling by the NMR ensemble is valid, and the quality of the NMR conformers is reasonable. MacArthur and Thornton [48] have found that protein cores observed in the NMR structures are well-defined and compare well with those of X-ray structures with a resolution of 2.0–2.3 Å. However, they have also observed that there is a greater disorder on the surface of NMR-derived protein structures. This may be due either to the inherent flexibility of the protein molecule in solution (as compared to the crystalline state), particularly of surface residues, or to fewer nOe restraints for surface residues. Philippopoulos and Lim [47] have compared an ensemble of *E. coli* ribonuclease H1 (RNase H1) conformers derived from NMR experiments with an ensemble derived from molecular dynamics simulations, as well as with two X-ray structures. They have shown that the NMR average structure is in better agreement with the high-resolution (1.48-Å) X-ray structure of the RNase H1 than with either the lower-resolution (2.05-Å) crystal structure or the molecular dynamics simulation. Furthermore, the 15 conformers of the NMR ensemble have sampled more conformational space of the RNase H1 than the 1.7-ns molecular dynamics simulations. The space covered by NMR structure ensembles overlaps significantly with that of ensembles generated in long molecular dynamics simulations [49]. NMR relaxation measurements can provide detailed experimental information on protein conformation dynamics in solution. The determination of ^{15}N relaxation parameters has become a routinely used method to characterize protein flexibility [50–52]. Using NMR relaxation methods, Lee et al. [53] have studied the dynamics of calcium-saturated calmodulin in the formation of a complex with a peptide model of smooth-muscle myosin light chain kinase. Their studies have resolved the motion of individual residues between the bound and unbound states and discriminated between backbone and side-chain perturbations. Here, we have used the NMR ensembles of conformers.

Salt bridges may be stabilizing or destabilizing to the protein, depending on three factors: the buried/exposed location of the ion-pairing residues in the protein structure; the geometrical orientation of the side-chain charged groups with respect to one another; and the interaction of the charged groups of the ion pair with the charged groups in the rest of the protein [17]. In particular, most of the ion pairs that obey the geometrical definition of a salt bridge, namely, 4.0 Å distance [3] between the charged group centroids and containing at least one pair of side-chain nitrogen and oxygen atoms within 4.0 Å distance, are stabilizing to the proteins. On the other hand, those with a distance exceeding a 4.0-Å limit largely contribute to destabilization of the protein structure. In the discussion below, we use the terms *salt bridge* and *ion pair* for the same set of charged residues. We refer to a pair of residues as a salt bridge if they satisfy the above definition. Otherwise, they are referred to as an ion pair.

Here, we study systemic flexibility in protein structure with the help of one specific example. We consider the salt bridge E68–K84 formed in the crystal structure of Cyanovirin-N (PDB code, 3ezm). Cyanovirin-N is a potent HIV-inactivating protein that inactivates diverse strains of the virus [28]. It was originally isolated from a cyanobacterium (blue-green algae). The primary structure of this protein consists of 101 amino acids organized into two domains. While in solution this protein exists in monomeric form [29], it invariably crystallizes as a domain-swapped dimer [28]. This indicates that this protein is highly flexible and hence a good candidate for studies of protein flexibility. The crystal structure of this protein has been solved to 1.5-Å resolution [28], and its NMR structure [29] ensemble contains 40 conformers. **Figures 1(a)** and **1(b)** respectively show the crystal structure of Cyanovirin-N and the superposition of the 40 NMR conformers of the same protein. Residues E68 and K84 form a salt bridge in the crystal structure of Cyanovirin-N. The side-chain charged group centroids are 3.94 Å apart, and the two side chains are inclined with respect to each other by 118°. The inclination angle is measured as the angle between two unit vectors. Each unit vector joins the C^{α} atom and the corresponding side-chain centroid in the charged residues constituting the salt bridge. In the NMR average minimized structure, the two side-chain charged group centroids are 5.17 Å apart, and the two side chains are inclined with respect to each other by 156°. In the 40 NMR conformers, the average distance between the side-chain charged group centroids is 5.90 ± 1.25 Å, and the average orientation of the charged-residue side chains (inclination angle) is 147.0 ± 20.3°. The locations of the two charged residues in the crystal structure, in the NMR average energy-minimized structure, and in the NMR conformer ensemble are given in **Table 3**. Residue E68 is buried to a greater extent in the crystal structure than in the NMR structure, while K84 has similar locations in the crystal and the NMR structures.

We have computed the strength of the electrostatic interactions between E68 and K84 in the crystal as well as in the 40 NMR conformers and in the average minimized conformer for Cyanovirin-N. **Figure 2** plots the variations in various energy terms in the 40 NMR conformers, along with the fluctuations in the location of the residues in the protein. It can be seen that the interactions between the two charged residues fluctuate between being stabilizing

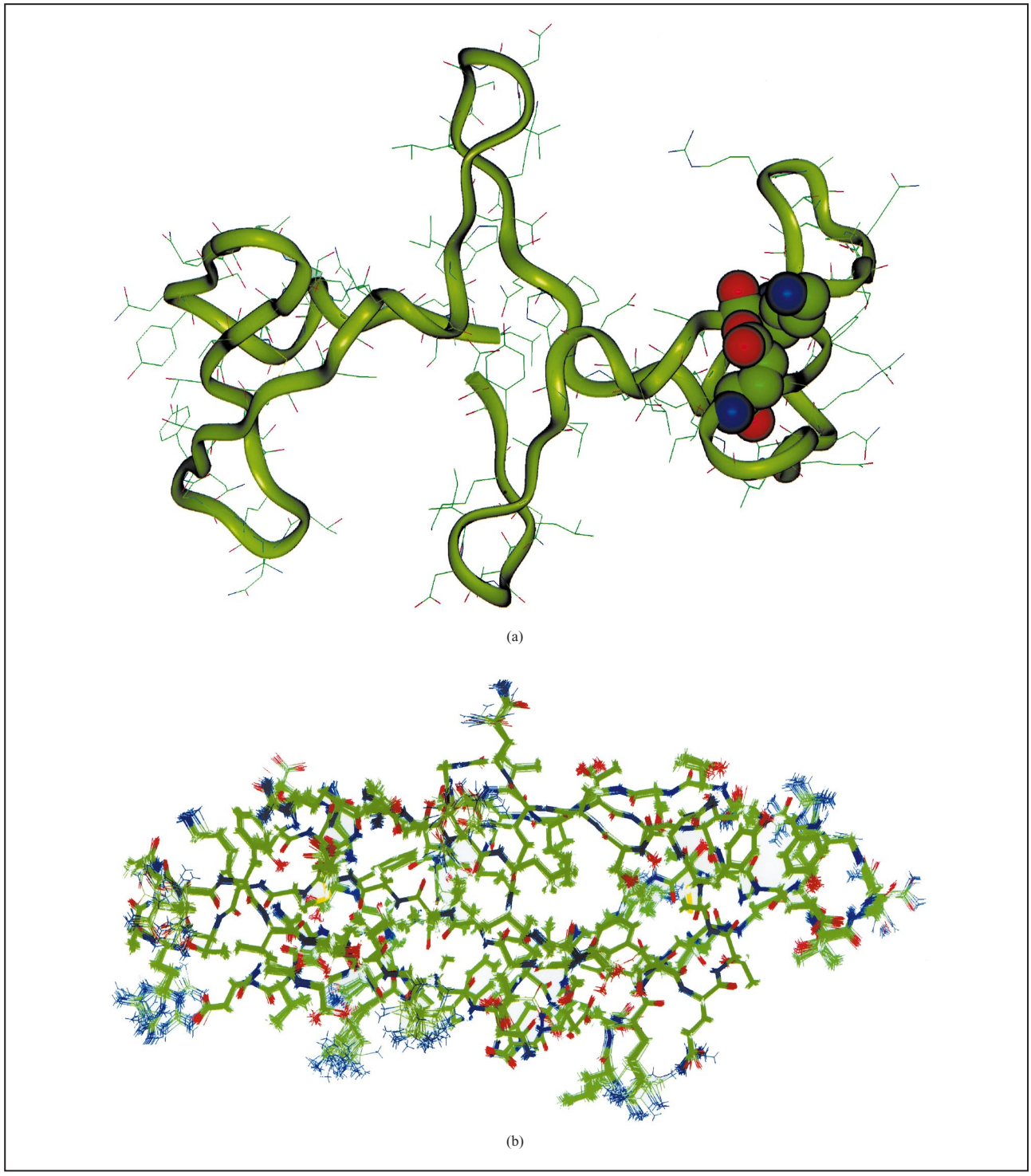

Figure 1

Structures of Cyanovirin-N determined by (a) X-ray crystallography and (b) nuclear magnetic resonance spectroscopy. In part (a), the green ribbon represents the C^{α} trace of Cyanovirin-N. Residues E68 and K84, which form a salt bridge in the crystal structure, are shown in CPK representation. In part (b), all 40 conformers in the NMR conformer ensemble are shown. For the sake of clarity, hydrogen atoms are not displayed. Part (a) was generated from the atomic coordinates of Cyanovirin-N crystal structure solved by Yang et al. [28] and available from the PDB [27] in file 3ezm. Part (b) was generated from the atomic coordinates of 40 NMR conformers of Cyanovirin-N. The NMR structure of Cyanovirin-N has been solved by Bewley et al. [29] and is available from the PDB [27] in file 2ezn.

Table 3 Geometries of the E68–K84 ion pair and the location of the residues E68 and K84 in crystal and NMR structures of Cyanovirin-N.

PDB entry	*Geometry*		*Location* [accessible surface area (%)]	
	r (Å)	*θ* (°)	*E68*	*K84*
3ezm (crystal structure)	3.94	118.1	16.7	67.3
2ezm (NMR average minimized structure)	5.17	156.0	40.0	66.3
2ezn (40 NMR conformers)	5.90 ± 1.25	147.0 ± 20.3	37.9 ± 6.6	66.1 ± 5.0

In the crystal structure, the residues E68 and K84 are close enough to be qualified as salt bridges. Note that E68 is buried to a greater extent in the protein crystal structure than in the NMR structure.

and destabilizing. In the crystal structure, salt bridge E68–K84 is destabilizing ($\Delta\Delta G_{tot}$ = +0.57 kcal/mol). In the average energy-minimized structure from the NMR conformer ensemble, the ion pair formed by the same residues is stabilizing ($\Delta\Delta G_{tot}$ = −0.51 kcal/mol). In the NMR conformer ensemble the strength of the interaction between the two residues varies between −1.21 and +4.09 kcal/mol. **Table 4** lists the values for the various energy terms for the interaction between E68 and K84 in Cyanovirin-N. On the whole, the electrostatic interactions between E68 and K84 in Cyanovirin-N appear to be weakly destabilizing. However, fluctuations in charged residue positions make this ion pair stabilizing in 25 of the 40 conformers and destabilizing in the remaining 15 conformers. The protein environment around the E68–K84 ion pair is destabilizing in eight conformers. The fluctuations in the electrostatic contribution of the E68–K84 ion pair in the NMR conformer ensemble are the result of fluctuations in the location of the two residues (Figure 2), as indicated by their accessible surface areas and the fluctuations in the orientation of the side-chain charged groups of these residues (**Figure 3**). Figure 3 shows that residues E68 and K84 repeatedly move close to each other and then apart. The two residues are within salt-bridging distance in the crystal structure of Cyanovirin-N. However, in the NMR conformer ensemble, the two residues remain within the salt-bridging distance only in five conformers: 4, 14, 29, 34, and 38. In the remaining 35 conformers, residues E68 and K84 are outside the salt-bridging distance.

While the salt bridge observed in the crystal structure of Cyanovirin-N is lost in most of the NMR conformers, another pair of charged residues, D44 and R76, come close to forming salt bridges in 14 conformers: 1, 2, 3, 11, 21, 24, 25, 27, 28, 29, 32, 33, 35, and 38. **Figure 4** shows this salt bridge. Conformers 29 and 38 contain both the salt bridges E68–K84 and D44–R76. Overall, 17 conformers in the NMR ensemble of Cyanovirin-N contain at least one salt bridge, and the remaining 23 conformers contain no salt bridge.

In summary, we have shown here that the systemic flexibility of Cyanovirin-N is reflected in the fluctuations of the strengths of the electrostatic interactions and the variability in the identity of the residues which form salt bridges in conformers of this protein. Further, these observations indicate that experimental observations with regard to formation or breakage of electrostatic interactions such as salt bridges depend on the relative conformer populations. Changes in experimental conditions, such as changes in temperature, pH, ionic strengths, the presence (or absence) of ligands, substrates, cofactors, or metal ions may result in a shift in the relative conformer populations [26]. Recently, we have studied fluctuations in electrostatic strengths of 22 salt bridges in 11 different proteins whose NMR conformer ensembles contain at least 40 conformers [54]. The trends observed in that large study are similar to those presented here for Cyanovirin-N, and hence substantiate its generality.

Conclusions

Electrostatic interactions are often regarded as specific interactions in proteins. On the other hand, nonpolar (hydrophobic) interactions are thought of as nonspecific, reflecting protein plasticity. Molecular flexibility is critical for protein function. However, too much flexibility leads to inspecific binding, and (in the more extreme cases) to instability, misfolding, and unfolding. Hydrophobic surfaces at the binding sites of proteins have been implicated in a broad range of ligand binding, typical of the less specific interactions. Excellent examples are those of the polyreactive antibodies [25].

Here we address the interrelationship between protein flexibility and electrostatic interactions. We consider both large- and small-scale motions. In large-scale motions, such as those observed in hinge-bending molecular movements, parts of the molecules, or entire subunits, move as rigid bodies with respect to other parts. On the other hand, smaller-scale motions reflect fluctuations around the native state. For the large-scale motions, we dissect the molecules into hydrophobic folding units. We

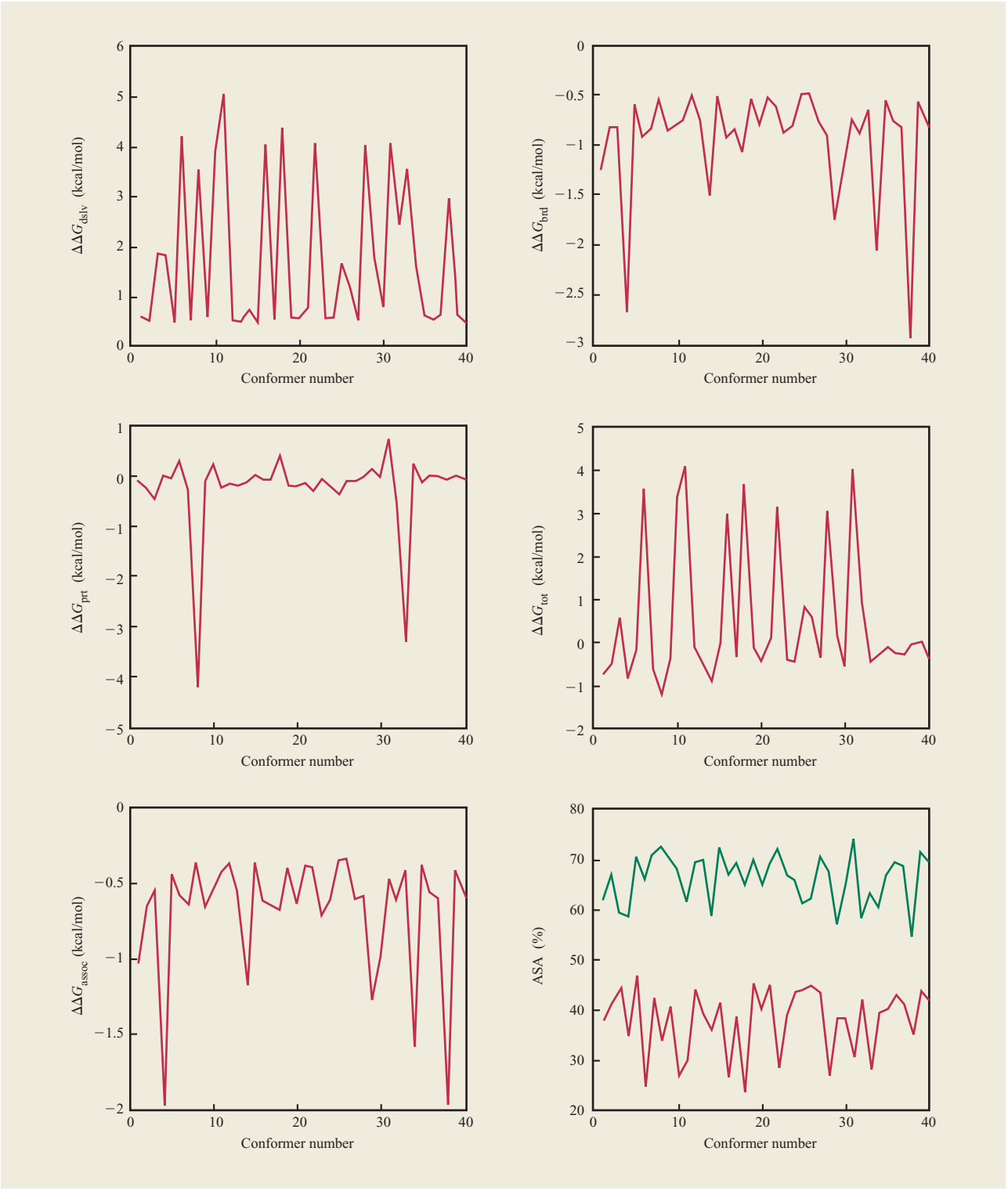

Figure 2

Conformer-dependent fluctuations in the stability of the E68–K84 ion pair in the NMR conformer ensemble of Cyanovirin-N (2ezn). The plots show the variations in all of the energy terms, namely $\Delta\Delta G_{dslv}$, $\Delta\Delta G_{brd}$, $\Delta\Delta G_{prt}$, $\Delta\Delta G_{tot}$, and $\Delta\Delta G_{assoc}$, and the accessible surface area [ASA (%)] for each residue, E68 (red) and K84 (green). The energy terms and ASA are described in the text. The ion pair E68–K84 shows extensive fluctuations in its stability. These fluctuations are the result of protein flexibility.

analyze the frequencies and calculate the stabilities of salt bridges formed between and within such stable units. In the sample we have studied, the electrostatic contributions to protein stability of salt bridges formed between hydrophobic folding units and those formed within them are of similar magnitudes. We note, however, that there are important differences in the component terms between salt bridges belonging to the intra- and inter-hydrophobic folding unit categories. On the other hand, there is a difference in the frequencies. The frequency of occurrence of salt bridges within the hydrophobic folding units is substantially greater than that observed between the units. This suggests that through evolution, stitching of the hydrophobic folding units with electrostatic interactions has been avoided, most likely since it hinders their motion, critical for protein function.

To address small-scale motions, conformer ensembles obtained by nuclear magnetic resonance experiments are particularly useful. Here, we have focused on the electrostatic interactions in Cyanovirin-N, an HIV-inactivating protein. Both NMR (40 conformers) and crystal structures are available for this protein. In the crystalline state, this protein is a domain-swapped dimer, while in solution it exists as a monomer. A salt bridge, formed by Glu68 and Lys84 and identified in the crystal structure of Cyanovirin-N, exists in only five conformers in the NMR conformer ensemble of Cyanovirin-N. In the remaining 35 conformers, the charged residues are beyond the salt-bridging limit. The electrostatic interaction between E68 and K84 fluctuates between being weakly stabilizing and weakly destabilizing. On the other hand, residues D44 and R76 of Cyanovirin-N come close enough to form salt bridges in 14 conformers. These residues do not form a salt bridge in the crystal structure of Cyanovirin-N. These observations indicate that the identity and strength of the observed electrostatic interactions in proteins are dependent upon their conformer populations.

Taken together, these computational observations illuminate some of the complex interrelationships between protein flexibility and electrostatic interactions. They illustrate that electrostatic interactions are sensitive to protein flexibility, since they depend upon the location and orientation of the interacting charges as well as their neighborhoods. Within the context of this issue of the *IBM Journal of Research and Development*, they further show the usefulness of computational analyses of protein structures in studies of aspects of the molecules which are vital to biological function: specificity, stability, and motion, and the role of electrostatics within this broad realm.

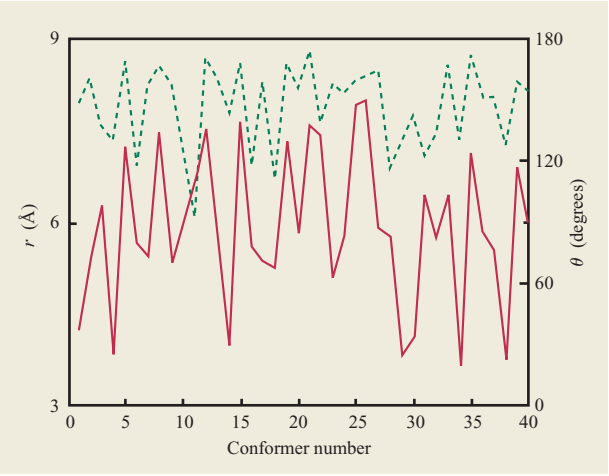

Figure 3

Conformer-dependent fluctuations in the geometry of ion pair E68–K84 in the NMR conformer ensemble of Cyanovirin-N. The plot shows the variations in the geometrical parameters r (Å) (solid curve) and θ (degrees) (dashed curve) for the ion pair, in different NMR conformers. The parameter r represents the distance between the centroids of the side-chain charged groups in E68 and K84. Only heavy (nonhydrogen) atoms were used in the computation of centroid positions. The parameter θ represents the angle of inclination of the two side-chain charged groups in E68 and K84. This angle is computed between two unit vectors. Each unit vector connects the C^{α} atom and the side-chain charged group centroid in each residue. From the figure it can be seen that the geometry of the E68–K84 ion pair fluctuates extensively in the NMR conformer ensemble.

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Table 4 Electrostatic strength of interaction between E68 and K84 in crystal and NMR structures of Cyanovirin-N.

Salt-bridge class	$\Delta\Delta G_{dslv}$ (kcal/mol)	$\Delta\Delta G_{brd}$ (kcal/mol)	$\Delta\Delta G_{prt}$ (kcal/mol)	$\Delta\Delta G_{tot}$ (kcal/mol)	$\Delta\Delta G_{assoc}$ (kcal/mol)
Crystal (3ezm)	+3.28	−1.65	−1.06	+0.57	−1.27
NMR average structure (2ezm)	+0.43	−0.90	−0.04	−0.51	−0.70
NMR conformer ensemble average (2ezn)	+1.72 ± 1.51	−0.95 ± 0.55	−0.26 ± 0.85	+0.52 ± 1.57	−0.68 ± 0.41

Note that the interaction is weakly destabilizing in the crystal structure. It is weakly stabilizing in the NMR average energy-minimized structure. However, the NMR conformer ensemble for 40 conformers indicates large fluctuations about the average value. These fluctuations in electrostatic strength of E68–K84 ion pairs are the outcome of "systemic" protein flexibility.

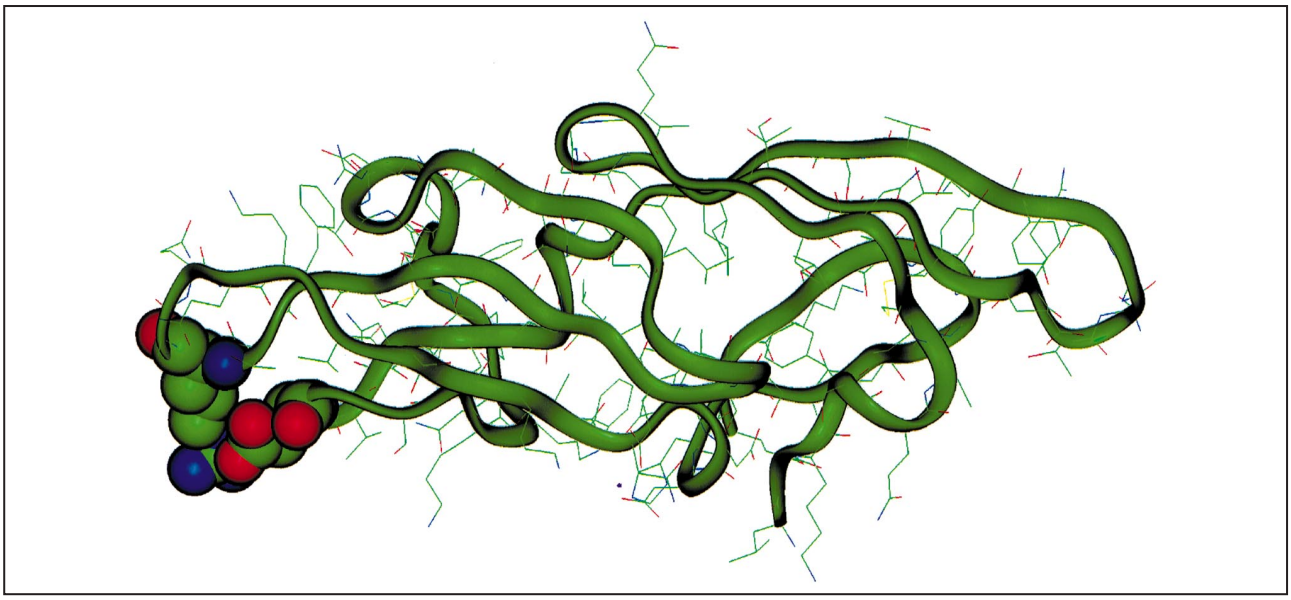

Figure 4

Conformer number 1 in the NMR conformer ensemble of Cyanovirin-N. In this conformer, residues E68 and K84, which form a salt bridge in the crystal structure [Figure 1(a)], are farther apart. Instead, another pair of charged residues (D44 and R76) are observed to be within salt-bridging distance. These residues are shown in CPK representation. The green ribbon represents the C^{α} trace of Cyanovirin-N. The residues E68 and K84 which form a salt bridge in the crystal structure of Cyanovirin-N do so in only five of the 40 conformers in the NMR conformer ensemble. On the other hand, residues D44 and R76 form salt bridges in 14 conformers. There are no salt bridges in 23 conformers in the ensemble. The NMR structure of Cyanovirin-N has been solved by Bewley et al. [29] and is available from the PDB [27] in file 2ezn.

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