# The Role of E27-K31 and E56-K10 Salt-Bridge Pairs in the Unfolding Mechanism of the B1 Domain of Protein G

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## ABSTRACT

*Molecular dynamics simulations of the B1 fragment of protein G (56 residues) have been performed at 325, 350, 375, 400, 450, and 500 K for 10 ns. An analysis of its structural and energetic parameters has indicated that the unfolding process of the GB1 protein begins at 900 ps of a 500 K simulation. The unfolding process is initiated when hydrogen bonds in the hydrophobic core region are broken; it continues with the α-helix transformation into coils and turns and ends with the destruction of the β-hairpins. These unfolding events are consistent with the hybrid model of the protein folding/unfolding mechanism, which is a compromise between the hydrophobic core collapse model and the zipper model. Salt-bridge pairs were found to play an important role in the unfolding process by maintaining the integrity of the tertiary structure of the protein. The breaking (or disappearance) of the salt-bridge pairs E27–K31 (in the α-helix) and E56–K10 (connecting $\beta_4$ and $\beta_1$) has resulted in the destruction of secondary structures and indicates the beginning of the unfolding process. Our results also suggest that the unfolding process in this simulation was not a complete denaturation of the protein because some β-hairpins remained.*

***Keywords***: *protein G; molecular dynamics; protein folding; hybrid model; salt-bridge pairs*

## ABSTRAK

*Simulasi dinamika molekul dari fragmen B1 protein G (56 residu) telah dilakukan pada suhu 325, 350, 375, 400, 450, dan 500 K selama 10 nanodetik. Analisis parameter struktural dan energetik mengindikasikan proses pembentangan protein GB1 dimulai saat waktu simulasi 900 pikodetik pada suhu 500 K. Proses pembentangan dimulai ketika ikatan-ikatan hidrogen pada inti hidrofobik protein rusak (putus), dilanjutkan dengan transformasi dari struktur α-heliks menjadi struktur coils dan turns, serta diakhiri dengan perusakan struktur β-hairpins. Proses pembentangan protein ini sesuai dengan model hibrida dari mekanisme pembentangan protein, yang merupakan kompromi dari model hydrophobic core collapse dan model zipper. Pasangan-pasangan jembatan garam diketahui memainkan peranan yang sangat penting dalam proses pembentangan protein dengan mempertahankan integritas dari struktur tersier protein. Pada penelitian ini diperoleh bahwa rusak atau putusnya pasangan jembatan garam E27-K31 (pada α-heliks) dan E56-K10 (menghubungkan struktur $\beta_4$ dan $\beta_1$) berakibat pada perusakan struktur sekunder yang menandai dimulainya proses pembentangan protein. Diperoleh pula bahwa proses pembentangan protein pada simulasi ini belum terjadi secara sempurna sebagaimana ditunjukkan oleh masih tersisanya struktur β-hairpins.*

***Kata Kunci***: *protein G; dinamika molekuler; pelipatan protein; model hibrida; pasangan jembatan garam*

## INTRODUCTION

Understanding the folding/unfolding process of proteins is vital since a protein's structure is very specific to its function. To function properly in biological processes, proteins must be folded into a three-dimensional structure. Misfolded structures are a source of diseases, like Alzheimer's, Parkinson's, and other health problems [1-3] the folding of proteins is a thermodynamic process in which the protein tries to find the most stable conformation that expends the least amount of energy, while for unfolding the opposite is true. By varying the temperatures of the protein-solvent system in the simulation, the structure evolution, dynamics, and stability of the protein can be determined.

In nature, the folding/unfolding process typically occurs on a time scale of micro- or milliseconds.

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Simulating this event is a challenging task and is also computationally expensive since the speed of the simulation is dependent on the size of the protein and the number of atoms involved. As an illustration, a typical all-atoms simulation of a 100-residue protein usually takes about a week for a several nanoseconds simulation run on a single computer (with a core i7 processor and 12 GB of RAM). Therefore, a simulation on a real-time scale (in microseconds or milliseconds) becomes unreachable and unfavorable. To overcome this problem, the temperature can be increased and the simulation time can be reduced [4]. This method does not change the nature of the unfolding process and still produces valid results [5].

The GB1 protein or the B1 domain of protein G (PDB code 1GB1) is a highly stable fold of the immunoglobulin binding domain that belongs to streptococcal protein G and helps *Streptomyces griseus* to evade the host's defenses [6]. This protein is quite interesting and has become a model used to explain the folding/unfolding process of proteins. Its simple structure and highly symmetric topology [7] combined with its small size, high thermal stability, and lack of a disulfide bridge [8] have made this protein (or its fragment, the β-hairpin) one of the most investigated proteins to explain the folding/unfolding mechanism. In biotechnology, the interest in this protein has also increased recently due to its applicability in medical areas. This protein has been used as a reagent for antibody affinity purification in the treatment of streptococcal infections [9]. The ability of the GB1 protein to immobilize antibodies has led to its use as an immunosensor because it is capable of controlling and manipulating the orientation of the antibody [10]. The stability of the β-hairpin structure of the B1 domain of protein G at high temperatures has been well studied using molecular dynamics (MD) simulations [5,11-15]. The simulation of the full 56-residues of the B1 domain of protein G has also been carried out by other researchers [16-21].

There are three models commonly used in explaining the folding/unfolding mechanism of proteins: the zipper model, the hydrophobic collapse model, and the hybrid model. In the zipper model [22], hydrogen bond interactions dominate the folding process [14-15,23], while in the hydrophobic collapse model [24], the folding process is led by hydrophobic interactions from the hydrophobic residues buried in the core of the protein [12,25]. The third model is the hybrid model [26], where both the hydrogen bond and hydrophobic interactions are responsible for the folding/unfolding process.

In the hybrid model, folding begins with the β-hairpin formation, followed by α-helix formation (coil to helix transition) and, finally, the formation of hydrogen bonds. The hydrophobic residues in the hybrid model act like a zipper to bring the hydrogen bonds in line before eventually locking the protein in its native state. For the unfolding process, the reverse steps are followed and begin with the breaking of backbone hydrogen bonds. Next, a helix to coil transition takes place, and the process ends with β-hairpin destruction.

This study aimed to identify the unfolding signatures and to reveal the unfolding mechanism of a GB1 protein and the role of salt-bridge pairs in maintaining the stability of the GB1 protein as the simulation temperature increased. Specific parameters were analyzed, including secondary structures, cartoon representations of the tertiary structures of the protein, Root Means Squared Fluctuations (RMSF), the number of hydrogen bonds, conformational energy, and salt-bridge pair electrostatic energy.

## EXPERIMENTAL SECTION

### Hardware

All simulations were carried out by a single CPU powered by a 3.4-GHz Intel® Core i7 processor with 12 GB of RAM and using the Ubuntu 12.04 Linux operating system.

### Software

The preparation of protein for the simulation was done using the Virtual Molecular Dynamics program (VMD) [27]. The MD simulations were performed using the Not (just) Another Molecular Dynamics (NAMD) v.2.9 simulator [28]. For the data analysis, the MD outputs (dcd file types) were analyzed using VMD to produce the output, such as RMSF, conformation energy, secondary structure, and cartoon representations of the protein's tertiary structure and hydrogen bonding. Some of the MD data were smoothed using the moving average method in Visual Basic for Applications (VBA), and Microsoft Excel before they were displayed here.

### Procedure

The structure of the B1 domain of protein G was taken from the Protein Data Bank with PDB code name 1GB1. The protein was prepared in a simulation box filled with the solvent atoms: a TIP3P water box [29] with a size of 64 Å x 54 Å x 50Å. Sodium and chloride counterions were used to neutralize the protein during the simulation. The MD simulation can be divided into three parts. The first part is minimizations. The second part is heating and equilibration, while the third part is the production run. A series of minimizations (four minimizations) was run for a total 100 ps. The

minimization process was intended to minimize the energy and relieve local strain from the initial structure of the system. The default method in the NAMD for energy minimization uses a conjugate gradient and a line search algorithm. After minimization, the system was gradually heated from 0 K to the desired simulation temperature (325, 350, 375, 400, 450, and 500 K) using 25 K increments every 20 ps. CHARM22 force fields that had been combined with the Particle Mesh Ewald (PME) and the Periodic Boundary Condition (PBC) methods with a 12.0-Å cutoff distance were used to calculate the interactions between atoms during the simulation. The heating process was carried out using an NVT ensemble. After heating, the system was then equilibrated twice to ensure that the structure was in stable condition. In the first equilibration, the system was constrained using the Langevin dynamics protocol for 20 ps to maintain the targeted simulation temperature. In the second equilibration (for 40 ps), the system was no longer constrained and was, therefore, free to move. The third part was the production run, where the protein was held at a constant temperature for 10 ns. Production runs were conducted in an NPT ensemble using Langevin dynamics (a Nosé-Hoover barostat and a Langevin's thermostat) to control the pressure and temperature of the system. Production runs were performed at 325, 350, 375, 400, 450, and 500 K using a 2.0-fs time step.

## RESULT AND DISCUSSION

### Signatures of Unfolding

The evolution of tertiary structures of proteins is depicted in tertiary structures representations of these proteins in Fig. 1. The MD simulations from 325 K up to 450 K did not reveal any significant change in the protein's tertiary structure. At a time of 900 ps and temperature of 500 K during the simulation, the unfolding process started to occur, as evidenced by the disappearance of the α-helix structure and some parts of the β-hairpins. Even though the tertiary structure of the protein had changed from its folded to an unfolded state, the overall tertiary structure still suggests a well-packed hydrophobic core, as can be seen from the robust structure of the β-hairpins and turns. Other researchers have also reported similar results [4].

The secondary structures of the protein at various temperatures are shown in Fig. 2. No significant change

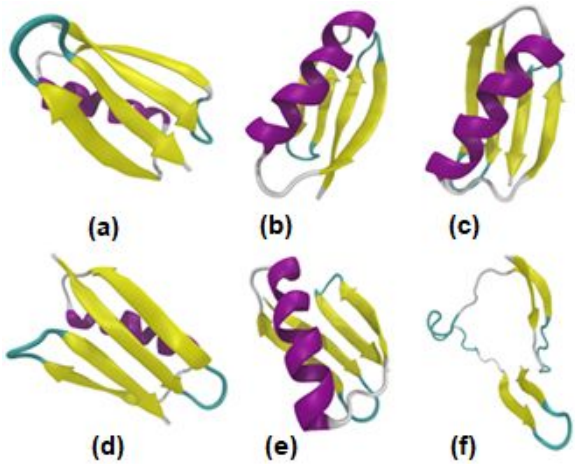


**Fig 1.** Model representations of the final tertiary structures (snapshots from the MD simulation) of the B1 domain of protein G at various simulation temperatures, at (a) T=325 K (b) T=350 K (c) T=375 K (d) T=400 K (e) T=450 K (f) T=500 K

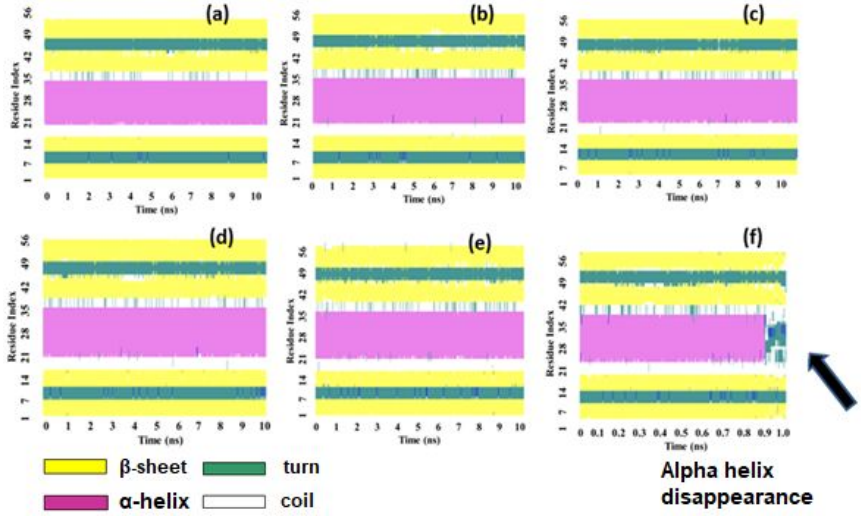


**Fig 2.** Change in the secondary structures at various simulation temperatures at (a) T=325 K (b) T=350 K (c) T=375 K (d) T=400 K (e) T=450 K (f) T=500 K. Note that the unfolding event is indicated by the disappearance of the α-helix structure, which was transformed into coil and turn structures

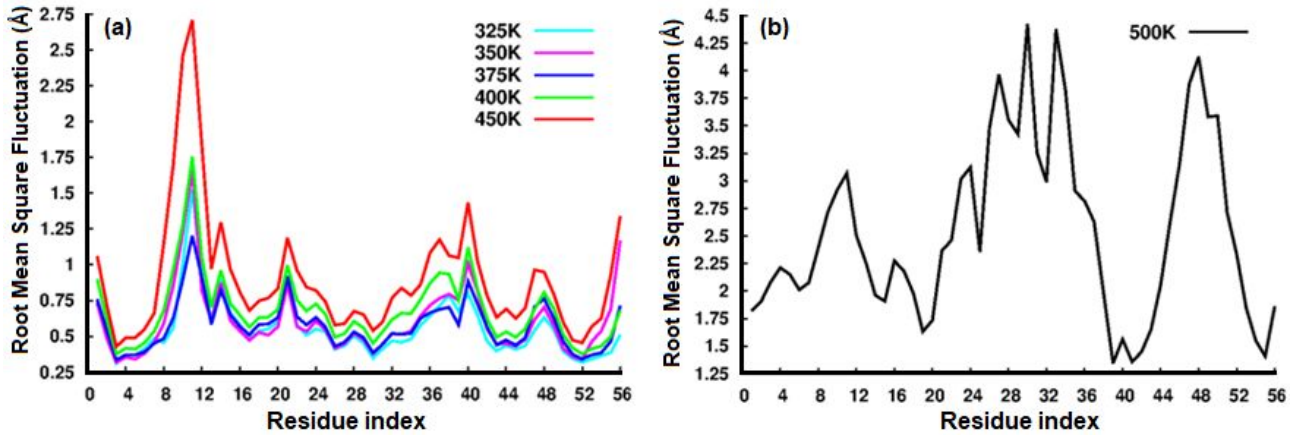


**Fig 3.** RMSF as a function of the residue index (number): (a) for temperatures of 325, 350, 375, 400, and 450 K, and (b) for 500 K. Note that there were flexible residues for simulation temperatures up to 450 K mostly in the coil and turn regions, while at 500 K they were shifted to the α-helix and β-hairpin regions

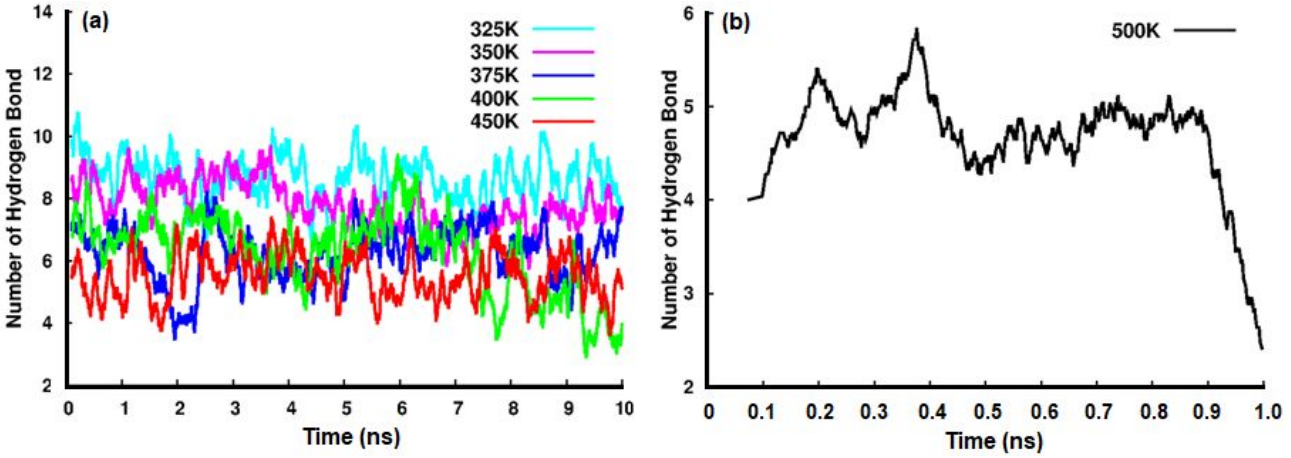


**Fig 4.** The number of hydrogen bonds as a function of the simulation time: (a) for temperatures of 325, 350, 375, 400, and 450 K, and (b) for 500 K. Note that at 500 K, most of the backbone hydrogen bonds have been broken

was observed at temperatures from 325 to 450 K. The unfolding signature of the GB1 protein occurred at its transition point of 900 ps at 500 K in the simulation, as indicated by the disappearance of the α-helix structure, which transformed into the coils and turns structures.

The RMSF values have revealed the flexibility of each residue during the simulation. The RMSF consistently increased as the temperature was raised. At 325, 350, 375, 400, and 450 K, the flexible residues were mostly found in the coil (V21, K28, D40) and turn (T11, A48) regions, as shown in Fig. 3a. In contrast, when unfolding occurred at 500 K, the flexibility was shifted in the secondary structures of the α-helix (A24, E27, F30, Y33) and β-sheet (K4, T16) regions (Fig. 3b). The increasing flexibility of the secondary structures marked the beginning of the transition process from a α-helix to the coil and turn structures that eventually destroyed the entire α-helix structure. We also found that of the five hydrophobic core residues in the β-hairpins region (L5, L7, G9, F52, V54), none showed a significant increase in flexibility at the transition point. This finding indicated that the breakage of hydrogen bonds might initiate the unfolding of the GB1 protein instead of the hydrophobic core destruction (collapse) process. The only hydrophobic core residue that became flexible at the transition point was F30 in the α-helix region; again, this finding emphasizes the transformation from a α-helix to a coil following the destruction of the β-hairpins. These results are in agreement with findings from other groups, where residues L5, F52, and V54 were considered to be among the most conserved residues in the GB1 protein and therefore somewhat difficult to break [30-31].

In the zipper model, the hydrogen bond is considered to be the dominant factor in the folding/unfolding process of proteins [22]. The backbone hydrogen bonds contribute to the β-hairpin stability of the protein's secondary structures [12]. The

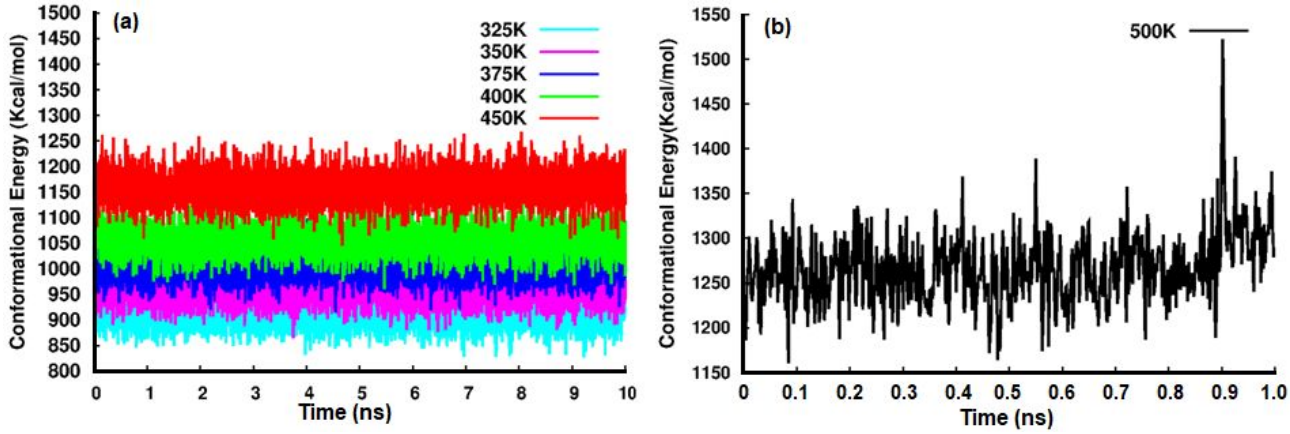


**Fig 5.** The conformational energy as a function of the simulation time (a) for temperatures of 325, 350, 375, 400, and 450 K; (b) for 500 K

rising simulation temperature increases the atomic vibration and also the total energy of the system, eventually leading to the breakage of the hydrogen bonds. As a consequence, the number of backbone hydrogen bonds decreases as the temperature is raised (Fig. 4a). From 325 to 450 K, the average number of backbone hydrogen bonds decreased from 10 to 5 bonds (a 50% decrease). At this transition point, most of the backbone hydrogen bonds have disappeared (only two bonds are left), which is expected for any protein that undergoes an unfolding event (Fig. 4b).

Early signs of unfolding were found during the 450 K simulation. At that temperature, the average number of backbone hydrogen bonds dropped by 50%, whereas the hydrophobic core residues (L5, L7, G9, A26, F30, A34, V39, F52, and V54) were rigid. These two findings indicate that the breakage of the hydrogen bonds precedes the hydrophobic core destruction (collapse) process. A similar conclusion obtained via steered molecular dynamics (SMD) simulations [32] also supports our findings. The existence of four flexible residues in the α-helix compared to only two in the β-hairpins explains why the secondary structures' destruction begins in the α-helix region rather than in that of the β-hairpins. Similar results that left the decomposition of the β-hairpins until the last step of the unfolding process have also been obtained by other researchers [4,30].

## Energetic Signatures of Unfolding and the Role of Salt-bridge Pairs

Conformational energy indicates the energy state of a protein in a particular conformation. The folded state corresponds to a low conformational energy, while an unfolded state is associated with a higher conformational energy. Conformational energy consists of binding, dihedral, torsion, and improper energy. The value of conformational energy increases as the simulation temperature is raised. In this study, the amount of conformational energy for the simulations from 325 to 450 K increased from about 850 to 1250 kcal/mol (Fig. 5a), but at 500 K, this energy reached 1520 kcal/mol (Fig. 5b). This sudden jump in conformational energy (around 300 kcal/mol) is related to the protein's drastic conformational change from a folded state into a more open (unfolded) state at its transition point.

During the simulation, the number of salt-bridge pairs in the protein was dynamic, but some salt-bridge pairs always exist at any temperature. These salt-bridge pairs (E56-K10 in the β-hairpins (connecting $\beta_4$ and $\beta_1$) and E27-K31 in the α-helix region) were continuously present at all simulation temperatures and were thought to offer a significant contribution to the stability of the protein. The breaking (or the disappearance) of those salt-bridges resulted in the destruction of the protein's secondary structures and the transformation of the protein's tertiary structure into an unfolded state, as shown in Fig. 6. The total energy released during these breakages is around 270 kcal/mol (approximately 100 kcal/mol for E56-K10 and 170 kcal/mol for the breaking of E27-K31). This drastic increase of about 300 kcal/mol in conformational energy during an MD simulation from 450 to 500 K matches the pair-breaking energy of the E56-K10 and E27-K31 salt-bridges and also emphasizes the importance of these salt-bridges in maintaining the stability of the protein in its folded state. The disappearance of E56-K10 in the β-sheet and E27-K31 in the α-helix destroys the secondary structures and accelerates the unfolding process.

An analysis of some of the protein's properties at various temperatures suggested that the unfolding transition satisfied a two-state folding kinetics model, which has also been suggested by other groups [22,33-35]. Even though the folding/unfolding equilibrium

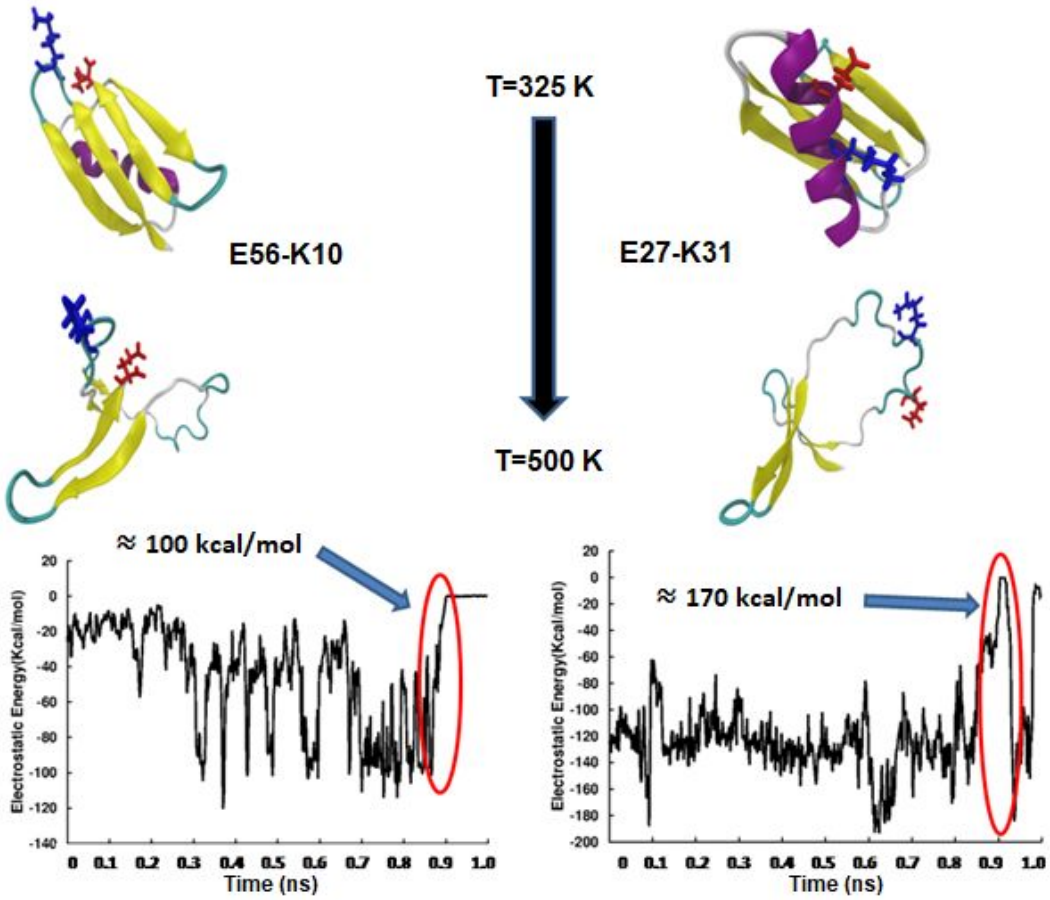


**Fig 6.** The tertiary structures representation of the E56-K10 and E27-K31 salt-bridge pairs breaking from a folded state at 325 K to an unfolded state at 500 K. The electrostatic energy for both salt-bridge pairs is a function of time at 500 K. Note that when the electrostatic energy becomes zero, the salt-bridge pair is broken and disappears from the system

thermodynamically supports the two-state model, recent developments suggest that the actual kinetics are far more complex and should also involve intermediate states [7,31,36]. Simulations under various solvent conditions (pH varied) [7] have confirmed the existence of such intermediate states.

## CONCLUSION

The MD simulation of the GB1 protein has been performed for 10 ns at various temperatures. The unfolding signatures were found at the transition point (T = 500 K and t = 900 ps) and were indicated by sudden changes in the structural and energetic parameters of the protein. Based on our simulation, we concluded that the unfolding of the GB1 protein occurs in the following order: (1) The unfolding begins with the drastic breakage of the backbone hydrogen bonds at 450 K. (2) The breaking of the E27-K31 salt-bridge pair at 500 K accelerates the helix-to-coil transition, while the breakage of the E56-K10 salt-bridge pair could initiate the beginning of the β-hairpin destruction process. At this transition point, the α-helix structure completely disappears, and the protein is transformed into the coil and turn structures. (3) The destruction of the β-hairpins is the last step in the unfolding process because the hydrophobic core residues buried in the β-hairpin regions are rigid and inflexible even at T = 500 K.

The aforementioned sequence of events supports a hybrid model of the folding/unfolding of proteins. β-hairpin destruction is the only unfinished step in our unfolding simulation. The hydrophobic core region (cluster) remains a well-packed structure, despite the increased exposure of this region to water molecules. A larger exposure of the hydrophobic core region to solvent molecules would assure the complete dissolution of β-hairpins and their full transformation into the turn and coil structures.

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