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Disruption and formation of surface salt bridges are coupled to DNA binding by integration host factor: a computational analysis

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Abstract

Revealing the thermodynamic driving force of protein/DNA interactions is crucial to the understanding of factors that dictate the properties and function of protein-DNA complexes. For the binding of DNA to DNA-wrapping proteins, such as the integration host factor (IHF), Record and co-workers have proposed that the disruption of a large number of pre-existing salt-bridges is coupled with the binding process (*J. Mol. Biol.*, 310, 2001, 379). To test this proposal, we have carried out explicit solvent MD simulations (multiple ~ 25–50 ns trajectories for each salt concentration) to examine the behavior of charged residues in IHF, especially concerning their ability to form salt bridges under different salt concentrations. Of the 17 cationic residues noted by Record and co-workers, most are engaged in salt-bridge interactions for a significant portion of the trajectories, especially in the absence of salt. This observation suggests that, from a structural point of view, their proposal is plausible. However, the complex behaviors of charged residues observed in the MD simulations also suggest that the unusual thermodynamic characteristics for IHF-DNA binding likely arise from the interplay between complex dynamics of charged residues both in and beyond the DNA binding site. Moreover, a comparison of MD simulations at different salt concentrations suggests that the large dependence of the IHF-DNA binding enthalpy on salt concentration may not be due to a significant decrease in the number of stable salt bridges in *apo* IHF at high salt concentration. In addition to the Hofmeister effects quantified in more recent studies of IHF-DNA binding, we recommend consideration of variation of the enthalpy change of salt bridge disruption at different salt concentrations. Finally, the current simulation study explicitly highlights that the electrostatic properties of DNA-binding proteins can be rather different in the *apo* and DNA bound states, which has important implication to the design of robust methods for predicting DNA binding sites in proteins.

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Supporting Information

The dependence of salt-bridge interactions on the salt concentration is explored with KCl solution as an example. Also included is the time dependence of salt bridge distances for all 36 cationic residues during MD simulations under no-salt and high-salt conditions. These materials may be accessed free of charge online at <http://pubs.acs.org>.

Protein-DNA complexes play multiple important roles in cellular processes that involve DNA “transactions”, such as replication, transcription, recombination and repair as well as the packaging of chromosomal DNA [1]. Proteins that interact with DNA in these complexes either serve as enzymes (e.g., DNA polymerase [2]) to catalyze biochemical reactions or simply act as an “architectural scaffold” [3] to manipulate the structure of DNA by, for example, bending and/or wrapping DNA. Well-known examples in the latter category include IHF [4,5] and HU [5]. Due to significant structural deviation from the equilibrium form of DNA (most often from the B-form), the mechanical work associated with DNA bending/wrapping needs to be compensated by the free energy gain of protein/DNA binding [6,7]. Therefore, it is important to understand the thermodynamic driving force of protein/DNA binding, especially for architectural scaffolding proteins.

IHF is a remarkable example for DNA-bending/wrapping proteins [4,5]. It bends the specific 34 bp DNA segment by as much as $\sim 160^\circ$, as illustrated by the crystal structure of an IHF-DNA complex [8] (see Fig.1a). To investigate the mechanism of IHF-DNA binding, Record and co-workers [9] explored extensively the thermodynamics of specific and non-specific interactions between IHF and DNA using isothermal titration calorimetry. These experiments revealed several unusual thermodynamic features of IHF-DNA interactions: i) the binding is highly exothermic and strongly enthalpy-driven while being entropically unfavorable; ii) the measured salt concentration dependence of the binding constant, SK_{obs} , is much smaller in magnitude than that estimated based on the oligocation model of the binding interface; iii) The enthalpy change ΔH_{sp}^o and heat capacity change ΔC_{sp}^o of the specific binding are strongly dependent on the salt concentration [KCl] and their magnitudes decrease significantly with increasing [KCl] between 100 mM and 350 mM.

To rationalize these unexpected features, Record and co-workers [9] proposed that IHF-DNA binding is coupled with the disruption of a large number of salt bridges that exist in the unbound form of IHF. In the crystal structure for the IHF-DNA complex [8], there are 23 cationic residues located within 6 Å from DNA phosphoryl groups [9]. This number is significantly larger than the average value (~ 12) found based on a large database of protein-DNA complexes [10]. Interestingly, there are also many anionic residues near these cationic residues. Based on an analysis of the position of these charged residues, Record and co-workers [9] suggested that 19 out of these 23 cationic residues can form salt bridges with nearby anionic residues following either side-chain rotations or backbone movements of flexible regions in the protein. Accordingly, they proposed that these salt bridges exist in the unbound form of IHF and get disrupted upon DNA binding and that disruption of salt bridges is the origin of the unusual thermodynamic features of the binding process. For example, the enthalpy change of salt bridge disruption is known to be negative [11], which explains the large exothermicity (enthalpic change) of the overall IHF-DNA binding process. They also proposed that the salt bridges become unstable at high $[K^+]$ due presumably to the screening effect of salt ions; the disruption of a reduced number of salt bridges during binding then decreases the magnitude of enthalpy change at high $[K^+]$, which was observed experimentally. Moreover, in a recent review article [12], Saecker and Record further speculated that the disruption of surface salt bridges coupled to DNA binding may not be unique to IHF and may occur in other DNA wrapping proteins as well, highlighting the possible generality of this phenomenon and its potential role in regulating DNA wrapping. Recent studies of Grove and co-workers [13,14] have pointed to the potential involvement of surface salt bridges in determining the length of DNA binding site in HU.

Since the proposal of salt bridge disruption was based on a structural analysis of the IHF-DNA complex, one may argue that the evidence is indirect without a high-resolution structure for IHF in the unbound state. For example, considering the small free energy gain associated with salt bridge formation at protein surface [15,16], it is not clear if a large

number (~23) of surface salt bridges would exist in the unbound state of IHF. Moreover, the simulation study of Elcock and co-workers [17] found that the stability of salt bridges is only marginally perturbed by salt concentration up to 2 M, thus the observed [KCl] dependence of IHF-DNA binding thermodynamics in experiments [9] may not be due to perturbations in the number of stable salt bridges. Indeed, more recent analysis of Vander Meulen et al. [18] that compared binding thermodynamics of IHF-DNA in different salt solutions (KCl, KF, KGlu) indicated that the large salt-dependence of the binding enthalpy is a Hofmeister effect arising from interaction of Cl^- with the surfaces buried in binding. Nevertheless, the extrapolated binding enthalpy at low salt is still highly negative, which is consistent with the salt-bridge disruption model.

These considerations have stimulated us to carry out molecular dynamics simulations to investigate salt bridge dynamics in IHF (see Fig.1b for snapshots). Considering the size of the system and complexity of the DNA binding process, we do not anticipate a quantitative comparison to experiments. The goal of the study is to explore whether the salt bridges proposed by Record and co-workers [9] are able to form and remain stable when IHF is not interacting with DNA. Along this line, we note that IHF has a large number of charged residues (36 cationic and 28 anionic residues out of 190 residues in total), thus it is interesting to investigate whether the salt bridges near the DNA binding site behave differently compared to other charged residues. In addition, we seek to investigate whether the propensity of salt bridge formation is perturbed by the presence of salt (K^+). Finally, since salt bridge formation/disruption is expected to significantly perturb electrostatic properties, which are used in many algorithms aimed at predicting DNA binding sites in proteins, it is of great interest to explore how sampling salt bridge dynamics impacts such predictions.

1 Computational Methods

1.1 Molecular dynamics simulations of IHF in the unbound state

Since the structure of the *apo* form of IHF is not available, we generate a model by simply removing DNA from the crystal structure of the IHF-DNA complex (PDB code 1IHF) [8]. Starting from this structure, MD simulations are carried out at two KCl concentrations: 0 mM (referred to as “no-salt”) and 350 mM (referred to as “high-salt”); 350 mM is chosen because it’s the highest salt concentration investigated in the experiments of Record and co-workers [9]. The simulations are performed using the GROMACS program [19,20] with the OPLS-AA force field [21] for IHF and ions. To set up the simulation, IHF is first solvated with a rhombic dodecahedron solvent box of TIP4P water [22] with $a = b = c = 100 \text{ \AA}$. The TIP4P water model is adopted due to its compatibility with the OPLS-AA force field. K^+ and Cl^- ions are then added into the simulation box according to the proper salt concentration. Since IHF bears a net charge of +8, 8 Cl^- ions are added in the no-salt system, and 149 K^+ and 157 Cl^- ions are added in the high-salt system, to achieve overall charge neutrality; snapshots of the two systems are shown in Fig.1. Periodic boundary condition is applied and long-range electrostatics are treated by the particle mesh Ewald method [23]. For van der Waals interactions, a switching cutoff scheme [24] for interatomic distances between 10 and 12 $\text{\AA}$ is used. The SETTLE [25] (for water) and SHAKE [26] (for protein) algorithms are applied to constrain bonds involving hydrogen atoms to allow an integration time step of 2 fs. The systems are equilibrated for 1 ns at 300K with the NVT ensemble, which is followed by 25–50 ns production runs with the NPT ensemble; three independent trajectories are carried out for each salt concentration. Temperature is controlled using the Nose-Hoover scheme [27,28] and pressure is controlled with the Berendsen scheme [29]. Considering the large number of charged residues in IHF (36 cationic and 28 anionic), we expect that three independent 25–50 ns trajectories are

sufficient for observing statistically meaningful behaviors of salt bridges (see Fig.4 and Supporting Information).

Since the system has many titratable residues, it is of interest to question whether many residues adopt unexpected protonation state. Considering the fairly large conformational changes during the simulation, we did not pursue computationally intensive pK_a analysis such as with MCCE [30]; this is in part justified by the observation that most titratable residues are solvent accessible and therefore not expected to have very large pK_a shift. This is confirmed by running pK_a calculations with the empirical PROPKA approach [31,32] with several different snapshots from the MD simulations; all Glu/Asp residues are predicted to be deprotonated and Lys/Arg protonated. Along the same line, the current work addresses salt-bridge interactions involving residues that are largely solvated rather than buried in the protein interior, thus the lack of explicit polarization in the force field is unlikely a serious issue. Recent analysis highlights that including polarization is most essential in problems that involve polar/non-polar interfaces [33–35].

1.2 Calculations of protein surface electrostatic potential

To compare the electrostatic properties of IHF in different structures, linear PB calculations are performed. Calculations are done using the PBEQ module in CHARMM [36] and the molecular surface is adopted to define the dielectric boundary between protein and solvent. Specifically, the set of atomic radii developed by Roux and co-workers [37] is used together with a solvent probe radius of 1.4 Å and an ion-exclusion layer of 2 Å. The partial charges for protein atoms are adopted from the standard CHARMM 27 force field [38]. The multigrid setup is used to solve PB numerically and the focusing scheme is applied: the coarser grid is $160\text{Å} \times 160\text{Å} \times 160\text{Å}$ with 1.0 Å spacing and the finer grid is $82\text{Å} \times 68\text{Å} \times 64\text{Å}$ with 0.4 Å spacing. The salt concentration is set as the physiological concentration of 0.15 M and the dielectric constant is set to be 2 and 80 for the protein and water, respectively. The electrostatic potential ϕ_i is first calculated for each grid point i in the finer grid enclosing the protein, which are then used to compute residue-averaged electrostatic potential (ϕ_{res}^j) using the PBAV option in CHARMM [36]. Specifically, ϕ_{res}^j is the average of ϕ_i over all grid points i that belong to a residue j (within the van der Waals radii of all atoms of residue j):

$$\phi_{res}^j = \frac{1}{N_j} \sum_{i,i \in j}^{N_j} \phi_i \quad (1)$$

where N_j is the number of grid points that belong to residue j .

1.3 Prediction of DNA binding interface

To investigate how change in electrostatic potentials (due to salt bridge rearrangements) impact prediction of DNA binding site (see Sect.2.3 for detailed discussions), we select two popular web servers that predict DNA binding interface in proteins. The first one is patch plus finder (PPF) [39] (<http://pfp.technion.ac.il/>), which finds the largest continuous patch on the protein surface that has positive electrostatic potential. The second one is Preds [40,41] (<http://pre-s.protein.osakau.ac.jp/preds/>), which predicts whether a protein is capable of binding DNA and, if so, location of the binding interface. The prediction is characterized by a statistical score, P_{score} , which is calculated based mainly on the surface electrostatic potential and the molecular shape of the protein. The only required input for both web servers is the protein structure in the standard PDB format.

2 Results and Discussions

Although three independent trajectories are calculated for each salt concentration, the results are qualitatively similar. Therefore, in the following discussions, unless explicitly stated, results for the longest simulation (50 ns) are discussed.

2.1 Overall conformational stability of IHF in the absence of DNA

Without the DNA, it is expected that the structure of IHF undergoes relaxation away from its conformation in the IHF-DNA complex (which is the starting configuration of the MD simulations). IHF is a heterodimer with two subunits, α and β , and can be divided into two domains according to secondary structure content and functional roles. The first domain (residues excluding 54 to 77 on both subunits, shown in green in Fig.2a) is made up of mainly α helices and is named as the core domain; the second “arm” domain (residue 54 to 77 on both subunits, shown in yellow in Fig.2a), on the other hand, consists mainly of β sheets and embrace the bound DNA in the IHF-DNA complex. As shown in Fig.2a, the core domain is relatively stable and the arm domain has more significant conformational changes during the MD simulations. To analyze the structural transitions more quantitatively, the RMSD of the backbone non-hydrogen atoms in the two domains are calculated along MD trajectories relative to the starting structure; the backbone non-hydrogen atoms of the core domain are used as the reference for best-fit in the RMSD calculations. As shown in Fig.2b, the RMSD of the core domain is ~ 2 Å throughout the 50 ns simulations at both no salt (0 mM KCl) and high-salt (350 mM KCl) conditions, which indicates that the core domain remains stable in the absence of DNA. The RMSD of the arm domain, however, can be as large as ~ 10 Å in both sets of trajectories, suggesting that the arm domain, which embraces DNA in the complex, becomes very floppy after DNA is removed. Moreover, the β -sheet structure in the arm domain (shown in purple in Fig.2a) undergoes partial unfolding during simulations, which hints at the possibility that IHF-DNA binding is coupled with local folding transition in the arm domain, which is often observed in the formation of specific protein-DNA complexes [42].

2.2 Salt bridge dynamics

In the proposal by Record and co-workers [9], 19 out of the 23 cationic residues in the DNA binding interface have the potential to (re)form salt bridges with nearby anionic residues in the absence of DNA. The potential salt bridge partners for two His residues (H54 and H79) on the β subunit are two anionic residues (D98 and D99) near the C-terminus of the α subunit, which is missing in the crystal structure of the IHF-DNA complex and therefore not included in our simulation. Thus, these two His residues are not included in the subsequent analysis, and we focus on the remaining 17 cationic residues (all are Lys or Arg); they are referred to as the 17 *binding-site* cationic residues. Moreover, as mentioned in the Introduction section, there are in total 36 cationic residues in IHF, thus we also explore the behaviors of the remaining 19 cationic residue in terms of the formation of salt bridge interactions.

2.2.1 Distribution of salt bridge distances—To analyze the propensity of all 36 cationic residues toward forming salt bridge interactions, we first monitor the shortest distance between the nitrogen atoms of each cationic residue and the oxygen atoms in all anionic residues (r_{min-NO}); as examples, the detailed data for all 36 cationic residues during the 50 ns trajectories are included in Supporting Information. To establish a distance criterion for defining salt bridge formation, a classical statistical study [43] concluded that $r_{min-NO} \leq 4$ Å should be used since this criterion leads to the largest difference in the frequency of observing oppositely charged pairs vs. likely charged pairs in protein crystal structures. In the analysis of Record and co-workers [9], however, $r_{min-NO} \leq 3$ Å was used

instead as a criterion for dehydrated (i.e. no solvent in between) salt bridges. To determine which criterion is more appropriate for the current study, we calculate the normalized histogram, $H(r_{min-NO})$, for all 36 cationic residues collected from both no-salt and high-salt simulations; the volume factor $4\pi r_{min-NO}^2$ is not included for simplicity. As shown in Fig.3, there is a large and sharp peak between 2 and 3.5 Å, which corresponds to the dehydrated salt bridge state, and another small peak (shown in the inset of Fig.3) between 3.5 and 5.5 Å, which corresponds to the solvent mediated ion pair. In a recent computational study [17] of acetate-methylammonium (model compounds for salt bridges) association, three minima were observed in the potential of mean force: one for the direct (dehydrated) interaction and another two for solvent separated interactions. The positions of the first two minima in that work are essentially the same as found here. The boundary between the two peaks in Fig.3 falls around 3.3 Å, therefore the distance criterion for defining a dehydrated salt bridge is chosen as $r_{min-NO} = 3.3$ Å, which is used in all subsequent analysis. This criterion is similar to that used by Record and co-workers [9] while more strict than that from the statistical study [43]. Comparison between the no-salt and high-salt results in Fig.3 suggests that increasing salt concentration decreases the probability of forming dehydrated salt bridges (the first peak) and the solvent-mediated ion pair (the second peak); as discussed in more detail below, however, the magnitude of the effect is rather small.

2.2.2 Salt bridge formation for the 17 binding-site cationic residues in the absence of KCl—

As shown in Fig.4 and figures in the Supporting Information, there is complex behavior in the salt-bridge dynamics. The salt bridge formation probability P for a specific cationic residue is defined as the fraction of simulation time during which this residue is engaged in a salt bridge interaction as determined based on the criterion $r_{min-NO} = 3.3$ Å; although we have only carried out three 25–50 ns trajectories for each salt concentration, there are significant fluctuations in the salt-bridge distances during the simulations for most cationic residues, thus the overall trends in P are expected to be meaningful; for example, the qualitative behaviors of the salt-bridges are fairly similar among the three independent trajectories. As shown in Table 1, P varies rather significantly between 0 and 1 for the 17 binding-site cationic residues discussed by Record and co-workers [9]. Nevertheless, the majority of these residues have formed salt bridges within 25–50 ns with considerable P values; this is true especially in the no-salt simulation. For example, if we (arbitrarily) choose $P = 0.1$ as the cutoff, 12 out of the 17 binding-site cationic residues form salt bridges in the no-salt simulation. This is quite remarkable since some of the cationic residues are rather far from any anionic residue in the starting (IHF-DNA complex) structure; the most striking example is R56β, which is 12.4 Å from any anionic residue in the initial structure but has a significant P value of ~0.3 in three independent trajectories (also see Fig.4d). Some salt bridges form quickly and remain stable throughout the simulation; for example, K20α, R55α (Fig.4a), K75β and R87β all have P values higher than 0.90 in at least one of the three independent trajectories. Out of the 17 binding site cationic residues, 5 of them (K45α, K57α, R60α, R59β and K81β) do not lead to significant salt bridge formation ($P < 0.01$) during any of the 25–50 ns of simulations; they are all rather far from anionic residues in the initial structure ($r_{min-NO} > 7.5$ Å), thus salt bridge formation likely implicates structural transitions at longer time scales (for their location in IHF, see Fig.5b). Nevertheless, even some of these residues come rather close to form stable salt-bridges during the simulations (e.g., see Fig.4b for R60α, for which r_{min-NO} decreases from ~14 to ~4 Å). In short, the simulations clearly illustrate that, in the absence of salt, a majority of the cationic residues in the DNA binding region has significant propensity to form salt bridges in the absence of DNA.

2.2.3 Salt bridge dynamics for the other 19 cationic residues—

Among other 19 cationic residues, which are further away from the DNA binding site in the IHF-DNA

complex structure, 11 of them are engaged in salt bridge interactions in the IHF-DNA complex (i.e., $r_{min-NO} < 3.3$ Å); all these, except K20β (see below), have high P values throughout the simulation. Among the rest, 5 of them (K15α, R21α, R77α, K97α, and K69β) also have significant P values (Table 2) although they are not engaged in salt bridges in the IHF-DNA complex. Therefore, the current simulation suggests that the disruption of salt bridges involving these cationic residues may also be coupled to the DNA binding process and contribute to the unusual thermodynamic features of IHF-DNA binding. These residues were not considered by Record and co-workers because they are more than 6 Å away from the phosphoryl oxygen atoms of DNA in the crystal complex structure [8]. Since electrostatic interactions are relatively long-ranged, it is not unreasonable that DNA binding can perturb interactions beyond the immediate binding region. On the other hand, to firmly support the involvement of these cationic residues, one needs to verify that these cationic residues do not engage in salt bridge interactions in an MD simulation of the IHF-DNA complex, which is beyond the scope of this work.

As alluded to above, K20β is somewhat unusual in that it forms salt bridges in the IHF-DNA complex structure but these salt bridges get disrupted completely in the absence of DNA in all three independent trajectories (see Table 2 and Supporting Information). This behavior is strikingly different from that of the other 35 cationic residues and motivates further structural analysis of this residue. The local environment of K20β at different stages of the simulation is shown in Fig. 6: in the initial structure, K20β forms bifurcated salt bridges with E23β and D24β; in the absence of DNA, as shown by a snapshot at 22.4 ns in the no-salt simulation (high-salt simulation shows similar behavior) in Fig. 6b, these two salt bridges get broken, and E23β and D24β form salt bridges with two of the 17 binding site cationic residues, K3β and K27β. Therefore, in addition to the disruption of salt bridges, there is also salt bridge switching (coupled disruption and formation) involved in the DNA binding process.

2.2.4 Effects of [KCl] on salt bridge formation—To explore the effect of KCl on salt bridge formation, we compare P for all 36 cationic residues under no-salt and high-salt conditions; since only three trajectories are run for each salt concentration, we show the P values from these simulations as separate data points in Fig. 5a rather than attempting to compute standard deviations. As shown in Fig. 5a, for most residues the effect of [KCl] is rather minor (also see Fig. 4a, c for R55α and K3β as representative examples). The exception is for four cationic residues in the DNA binding region: R76α, K88α, R56β, and R87β. These four residues (shown in bold in Table 1 and in yellow in Fig. 5b) have considerable P values under no-salt condition but essentially zero P value with 350 mM KCl. A common feature for these four residues is that they are relatively far from anionic residues in the starting structure (see Supporting Information for details); the shortest r_{min-NO} is for R87β, which is 5.9 Å. As shown in Fig. 4d for R56β, although r_{min-NO} is seen to decrease substantially from the initial (crystal) value of 12.4 Å to salt-bridge range (< 3.3 Å) in the no-salt simulation, the distance rarely decreases to below 5 Å in the high-salt simulation. Therefore, the overall trend is that KCl has a significant impact on salt bridge formation only if the charged residues are initially far away from each other. This trend makes physical sense since the screening effect associated with salt ions only plays a significant role at long range. At short distances (compared to the size of solvent), electrostatic screening is not expected to be important. For example, the computational study of Elcock and co-workers [17] on the association of acetate and methylammonium (model compounds for salt bridges) found that the free energy minimum that corresponds to the dehydrated salt bridge state does not diminish even up to 2 M salt concentration. As the salt concentration increases from 0 to 0.3 M (similar to the “high-salt” condition here), the stability of the dehydrated salt bridge is reduced by only 0.2 kcal/mol.

These analyses suggest that even at [KCl] of 350 mM, a significant fraction of salt bridges in the unbound form of IHF is expected to be stable (8 out of 17 residues have P_{salt} larger than 0.1 in at least one 25–50 ns trajectory). This conclusion seems to be inconsistent with the proposal of Record and co-workers that salt bridges are unstable at 350 mM $[K^+]$ and hence that there is no disruption of salt bridges coupled to DNA binding. This second hypothesis was proposed to explain the experimental observation that ΔH_{sp}^o for specific binding increases (becomes less negative) significantly from -56.1 kcal/mol at 60 mM $[K^+]$ to -10.8 kcal/mol at 350 mM $[K^+]$ at 40°C. More recent analyses [18], however, found that a significant portion of the large salt dependence is due to the Hofmeister effect associated with Cl^- ; extrapolations of results in different salt solutions (KCl, KF, and KGlu) found that, at low salt concentration, the binding enthalpy is essentially independent of the salt concentration. Nevertheless, it is of interest to discuss other factors that can potentially contribute to the salt dependence of binding thermodynamics in light of the current simulations. For example, since our simulation results indicate that the disruption of multiple salt bridges is still likely involved in DNA binding at even 350 mM $[K^+]$, we suspect that the increase of ΔH_{sp}^o is due, at least in part, to the increase of the enthalpy change of disrupting a salt bridge (ΔH_d) with respect to the increase of $[K^+]$. This can be understood by comparing the processes of salt bridge disruption at no-salt and high-salt conditions. The enthalpy change for salt bridge disruption at the no-salt condition ($\Delta H_d^{no-salt}$) is negative [44] due to the gain of hydration enthalpy when two charged species are separated. At higher salt concentration, however, the disruption of a salt bridge is coupled to the formation of two side-chain-counterion interaction pairs. As a result, the enthalpy change (ΔH_d^{salt}) should be $\Delta H_d^{no-salt}$ plus the enthalpy change of forming two side-chain-counterion pairs, which is positive and therefore ΔH_d^{salt} should be larger (less negative) than $\Delta H_d^{no-salt}$; at a qualitative level, this effect is embedded with the calculations for the enthalpy change for K^+-Cl^- ion-pair dissociation at different concentrations (see Supporting Information). Whether this effect is important at a quantitative scale remains to be determined because the estimated impact for K^+-Cl^- ion-pair dissociation is fairly modest in magnitude (see Supporting Information); moreover, this contribution can be offset by similar effects, but of the opposite sign, associated with the formation of ionic interactions between the cationic residues in IHF and phosphoryl groups in DNA.

2.3 Impact on the prediction of DNA binding sites

As mentioned in the Introduction section, an important goal of this study is to explore how sampling salt bridge dynamics impacts the prediction of DNA binding sites on proteins. The goal is motivated by the consideration that salt bridge formation/disruption is expected to significantly modulate the system electrostatics, which play a major role in many DNA binding site prediction algorithms [39–41,45–47]. Indeed, many such algorithms have been tested or trained using the structures of protein-DNA complexes (with the DNA excised). If there is significant structural difference between the unbound and bound conformations of the protein, such as the formation/disruption of a large number of salt bridges, the electrostatic properties may differ significantly. As a result, an algorithm trained using bound-state structures may not be effective since realistic applications will require using the unbound state as input.

To illustrate the change of surface electrostatic potential in IHF due to salt bridge dynamics, we calculate the differential electrostatic potential between IHF adopting the conformation in the IHF-DNA complex (PDB code 1IHF [8]) and a snapshot from the no-salt simulation in which the maximum number (12 out of 17) of binding-site cationic residues have formed salt bridges (see Fig.7). For the red regions in Fig.7, the residue-averaged electrostatic potential difference ($\Delta\phi_{res}$) is more than $5 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{e}^{-1}$; $\Delta\phi_{res}$ is less than 5

kcal·mol⁻¹·e⁻¹ for the blue regions whereas it is between -5 and 5 kcal·mol⁻¹·e⁻¹ for the white regions. It is clear that for most of the DNA binding interface, the electrostatic potential is decreased by more than 5 kcal·mol⁻¹·e⁻¹ as the IHF structure relaxes away from the conformation in the IHF-DNA complex. We emphasize here that the structural variations sampled in this study are limited to the time scale of 25–50 ns; with the realistic structure of the *apo* state IHF, the difference in the structural and electrostatic properties can be even greater.

The key question is whether such change in the surface electrostatic potential has a significant impact on the performance of structure-based algorithms for DNA binding site prediction. As discussed in the **Computational Methods** section, we test this using two popular structure-based web servers. The first server is patch plus finder (PPF) [39], which finds the largest continuous patch with positive electrostatic potential on the protein surface [47]. Using the IHF structure in the IHF-DNA complex as input, the predicted patch by PPF (see Fig.8a) contains 63 residues and coincides with the authentic DNA binding interface. Using the snapshot with altered salt bridge patterns as the input structure, however, the predicted patch (see Fig.8b) contains 35 residues and spans only half of the arm domain.

The second server we have tested is Preds [40,41], which makes prediction based on not only surface electrostatic potential but also molecular shape features. An output of Preds is a statistical score, P_{score} , and the input protein is predicted to be a DNA binding protein if P_{score} is higher than 0.12. Using the IHF structure in the IHF-DNA complex as input, the predicted P_{score} is 0.27; the score becomes substantially lower (0.17) when the snapshot from simulation is used. Another output of Preds is the predicted binding sites, which are shown in Fig.8c and 8d with different IHF structures as input; the difference is relatively small, and the predicted binding site only reduces from 90 to 73 residues when the snapshot from MD simulation is used instead of the IHF structure in the IHF-DNA complex. Therefore, the prediction of DNA binding site appears to be more robust when electrostatic properties are augmented by geometrical considerations.

3 Concluding Remarks

Protein-DNA binding processes likely involve many structural transitions at both domain and side-chain levels, which are not be straightforward to capture using experiments alone. In this study, motivated by the proposal of Record and co-workers in recent thermodynamic studies of specific DNA binding to IHF [9], we have focused on the behavior of charged residues in IHF using explicit solvent molecular dynamics simulations. Even at the somewhat limited time scale of 25–50 ns, we have observed rather rich behaviors. Of the 17 cationic residues noted by Record and co-workers, most are engaged in salt-bridge interactions for a significant portion of the trajectories, especially under the no-salt condition. This observation suggests that, from a structural point of view, their proposal that IHF-DNA binding is coupled with the disruption of pre-existing salt-bridges in *apo*-IHF is plausible. However, switching of salt bridge pairs, i.e. the simultaneous disruption and formation of salt bridges, that involve non-binding site cationic residues has also been observed during the MD simulations. Therefore, the unusual thermodynamic characters for IHF-DNA binding likely arise from the interplay between fairly complex dynamics of charged residues both in and beyond the DNA binding site. Regarding the salt effect, the MD simulations suggest that it only contributes to the screening of electrostatic interactions when salt bridge pairs are far away from each other, consistent with a recent computational study of small molecules that form salt bridges [17]. Therefore, the large dependence of the IHF-DNA binding enthalpy on salt concentration may not be due to a significant decrease in the number of stable salt bridges in *apo* IHF at high salt concentration; this is qualitatively

consistent with more recent analysis [18], which found that the salt dependence of binding enthalpy is largely a Hofmeister effect.

Our simulation study further highlights that the electrostatic properties of DNA-binding proteins can be rather different in the *apo* and DNA bound states. This has important implications to the design of methods for predicting DNA binding sites in proteins. As we have illustrated using two examples, it appears essential to either explicitly consider sampling salt-bridge dynamics when evaluating electrostatic properties, and/or to supplement electrostatic properties with geometrical criteria based on structural complementarity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HU	histone-like protein from <i>E. coli</i> strain U93
IHF	integration host factor
MD	Molecular Dynamics
PB	Poisson-Boltzmann
RMSD(F)	root mean square difference (fluctuation)

References

1. Alberts, B.; Bray, D.; Lewis, J.; Raff, M.; Roberts, K.; Watson, JD. Molecular biology of the cell. Garland Publishing, Inc.; 1994.
2. McHenry CS. DNA polymerase-III holoenzyme of Escherichia-coli. Annu. Rev. Biochem. 1988; 57:519–550. [PubMed: 3052282]
3. Bewley CA, Gronenborn AM, Clore GM. Minor groove-binding architectural proteins: Structure, function, and dna recognition. Annu. Rev. Biophys. Biomol. Struct. 1998; 27:105–131. [PubMed: 9646864]
4. Rice PA. Making DNA do a U-turn: IHF and related proteins. Curr. Opin. Struct. Biol. 1997; 7:86–93. [PubMed: 9032059]
5. Swinger KK, Rice PA. IHF and HU: flexible architects of bent DNA. Curr. Opin. Struct. Biol. 2004; 14:28–35. [PubMed: 15102446]
6. Williams LD. Electrostatic mechanisms of DNA deformation. Annu. Rev. Biophys. Biomol. Struct. 2000; 29:497–521. [PubMed: 10940257]
7. Ma L, Yethiraj A, Chen X, Cui Q. A computational framework for mechanical response of macromolecules: application to the salt concentration dependence of DNA bendability. Biophys. J. 2009; 96:3543–3554. [PubMed: 19413960]
8. Rice PA, Yang SW, Mizuuchi K, Nash HA. Crystal structure of an IHF-DNA complex: A protein-induced DNA U-turn. Cell. 1996; 87:1295–1306. [PubMed: 8980235]

9. Holbrook JA, Tsodikov OV, Saecker RM, Record MT. Specific and non-specific interactions of integration host factor with DNA: Thermodynamic evidence for disruption of multiple IHF surface salt-bridges coupled to DNA binding. *J. Mol. Biol.* 2001; 310:379–401. [PubMed: 11428896]
10. Nadassy K, Wodak SJ, Janin J. Structural features of protein-nucleic acid recognition sites. *Biochemistry.* 1999; 38:1999–2017. [PubMed: 10026283]
11. Perl D, Mueller U, Heinemann U, Schmid FX. Two exposed amino acid residues confer thermostability on a cold shock protein. *Nat. Struct. Biol.* 2000; 7:380–383. [PubMed: 10802734]
12. Saecker RM, Record MT. Protein surface salt bridges and paths for DNA wrapping. *Curr. Opin. Struct. Biol.* 2002; 12:311–319. [PubMed: 12127449]
13. Grove A, Saavedra TC. The role of surface-exposed lysines in wrapping DNA about the bacterial histone-like protein HU. *Biochem.* 2002; 41:7597–7603. [PubMed: 12056890]
14. Grove A. Surface salt bridges modulate DNA wrapping by the type II DNA-binding protein TF1. *Biochem.* 2003; 42:8739–8747. [PubMed: 12873134]
15. Daopin S, Sauer U, Nicholson H, Matthews BW. Contributions of engineered surface salt bridges to the stability of t4 lysozyme determined by directed mutagenesis. *Biochem.* 1991; 30:7142–7153. [PubMed: 1854726]
16. Strop P, Mayo SL. Contribution of surface salt bridges to protein stability. *Biochem.* 2000; 39:1251–1255. [PubMed: 10684603]
17. Thomas AS, Elcock AH. Direct observation of salt effects on molecular interactions through explicit-solvent molecular dynamics simulations: Differential effects on electrostatic and hydrophobic interactions and comparisons to poisson-boltzmann theory. *J. Am. Chem. Soc.* 2006; 128:7796–7806. [PubMed: 16771493]
18. Meulen KAV, Saecker RM, Record MT. Formation of a wrapped DNA-protein interface: Experimental characterization and analysis of the large contributions of ions and water to the thermodynamics of binding IHF to H' DNA. *J. Mol. Biol.* 2008; 377:9–27. [PubMed: 18237740]
19. Lindahl E, Hess B, van der Spoel D. Gromacs 3.0: a package for molecular simulation and trajectory analysis. *J. Mol. Model.* 2001; 7:306–317.
20. Berendsen HJC, Vanderspoel D, Vandrunen R. Gromacs - a message-passing parallel molecular-dynamics implementation. *Comput. Phys. Commun.* 1995; 91:43–56.
21. Jorgensen WL, Tiradorives J. The OPLS potential functions for proteins - energy minimizations for crystals of cyclic-peptides and crambin. *J. Am. Chem. Soc.* 1988; 110:1657–1666.
22. Jorgensen WL, Chandrasekhar J, Madura JD, Impey RW, Klein ML. Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* 1983; 79:926–935.
23. Essmann U, Perera L, Berkowitz ML, Darden T, Lee H, Pedersen LG. A smooth particle mesh ewald method. *J. Chem. Phys.* 1995; 103:8577–8593.
24. Steinbach PJ, Brooks BR. New spherical-cutoff methods for long-range forces in macromolecular simulation. *J. Comput. Chem.* 1994; 15:667–683.
25. Miyamoto S, Kollman PA. Settle - an analytical version of the shake and rattle algorithm for rigid water models. *J. Comput. Chem.* 1992; 13:952–962.
26. Ryckaert JP, Cicciotti G, Berendsen HJC. Numerical-integration of cartesian equations of motion of a system with constraints - molecular-dynamics of n-alkanes. *J. Comput. Phys.* 1977; 23:327–341.
27. Nose S. A unified formulation of the constant temperature molecular-dynamics methods. *J. Chem. Phys.* 1984; 81:511–519.
28. Hoover WG. Canonical dynamics - equilibrium phase-space distributions. *Phys. Rev. A.* 1985; 31:1695–1697. [PubMed: 9895674]
29. Berendsen HJC, Postma JPM, Vangunsteren WF, Dinola A, Haak JR. Molecular-dynamics with coupling to an external bath. *J. Chem. Phys.* 1984; 81:3684–3690.
30. Gunner MR, Mao J, Song Y, Kim J. Factors influencing the energetics of electron and proton transfers in proteins. what can be learned from calculations? *Biochim. Biophys. Acta.* 2006; 1757:942–968. [PubMed: 16905113]
31. Li H, Robertson AD, Jensen JH. Very fast empirical prediction and interpretation of protein pK_a values. *Proteins: Struct., Funct., and Bioinf.* 2005; 61:704–721.

32. Bas DC, Rogers DM, Jensen JH. Very fast prediction and rationalization of pK_a values for protein-ligand complexes. *Proteins: Struct., Funct., and Bioinf.* 2008; 73:765–783.
33. Leontyev IV, Stuchebrukhov AA. Electronic continuum model for molecular dynamics simulations of biological molecules. *J. Chem. Theo. Comp.* 2010; 6:1498–1508.
34. Harder E, MacKerell AD Jr, Roux B. Many-body polarization effects and the membrane dipole potential. *J. Am. Chem. Soc.* 2009; 131:2760–2761. [PubMed: 19199514]
35. Jiao D, Golubkov PA, Darden TA, Ren P. Calculation of protein-ligand binding free energy by using a polarizable potential. *Proc. Natl. Acad. Sci. USA.* 2008; 105:6290–6295. [PubMed: 18427113]
36. Brooks BR, Bruccoleri RE, Olafson BD, States DJ, Swaminathan S, Karplus M. Charmm - a program for macromolecular energy, minimization, and dynamics calculations. *J. Comput. Chem.* 1983; 4:187–217.
37. Nina M, Im W, Roux B. Optimized atomic radii for protein continuum electrostatics solvation forces. *Biophys. Chem.* 1999; 78:89–96. [PubMed: 17030305]
38. MacKerell AD, Bashford D, Bellott M, Dunbrack RL, Evanseck JD, Field MJ, Fischer S, Gao J, Guo H, Ha S, Joseph-McCarthy D, Kuchnir L, Kuczera K, Lau FTK, Mattos C, Michnick S, Ngo T, Nguyen DT, Prodhom B, Reiher WE, Roux B, Schlenkrich M, Smith JC, Stote R, Straub J, Watanabe M, Wiorkiewicz-Kuczera J, Yin D, Karplus M. All-atom empirical potential for molecular modeling and dynamics studies of proteins. *J. Phys. Chem. B.* 1998; 102:3586–3616.
39. Shazman S, Celniker G, Haber O, Glaser F, Mandel-Gutfreund Y. Patch Finder Plus (PFplus): A web server for extracting and displaying positive electrostatic patches on protein surfaces. *Nucleic Acids Res.* 2007; 35:W526–W530. [PubMed: 17537808]
40. Tsuchiya Y, Kinoshita K, Nakamura H. PreDs: a server for predicting dsDNA-binding site on protein molecular surfaces. *Bioinformatics.* 2005; 21:1721–1723. [PubMed: 15613393]
41. Tsuchiya Y, Kinoshita K, Nakamura H. Structure-based prediction of DNA-binding sites on proteins using the empirical preference of electrostatic potential and the shape of molecular surfaces. *Proteins-Struct. Funct. Bioinf.* 2004; 55:885–894.
42. Spolar RS, Record MT. Coupling of local folding to site-specific binding of proteins to DNA. *Science.* 1994; 263:777–784. [PubMed: 8303294]
43. Barlow DJ, Thornton JM. Ion-pairs in proteins. *J. Mol. Biol.* 1983; 168:867–885. [PubMed: 6887253]
44. Kauzmann W. Some factors in interpretation of protein denaturation. *Adv. Prot. Chem.* 1959; 14:1–57.
45. Gou, Z.; Hwang, S.; Kuznetsov, BI. Sequence-based prediction of DNA-binding sites on DNA-binding proteins. *Proceedings of the Fifth International Conference on Bioinformatics of Genome Regulation and Structure I*; 2006. p. 268-271.
46. Tjong H, Zhou HX. DISPLAR: an accurate method for predicting DNA-binding sites on protein surfaces. *Nucleic Acids Res.* 2007; 35:1465–1477. [PubMed: 17284455]
47. Stawiski EW, Gregoret LM, Mandel-Gutfreund Y. Annotating nucleic acid-binding function based on protein structure. *J. Mol. Biol.* 2003; 326:1065–1079. [PubMed: 12589754]
48. Humphrey W, Dalke A, Schulten K. VMD - Visual Molecular Dynamics. *J. Mol. Graph.* 1996; 14:33–38. [PubMed: 8744570]

Figure 1.

Structure of IHF. (a) The crystal structure of IHF-DNA complex (pdb 1IHF). IHF is shown in New Cartoon and in purple while DNA is shown in Ribbons and in yellow. The 17 proposed cationic residues by Record and co-workers (not including two His residues, H54 and H79) are shown in Licorice. (b) A snapshot from MD simulations of *apo*-IHF at 350 mM [KCl]. The starting structure is obtained from the crystal structure of the IHF-DNA complex (PDB code 1IHF [8]), from which the DNA is removed. IHF is shown in the Cartoon scheme and in purple; oxygen atoms of water molecules are shown as points and in iceblue. K⁺ and Cl⁻ ions are shown as spheres and in yellow and green, respectively. The figures are made using VMD. [48]

Figure 2.

Conformational transitions in IHF observed within 50 ns MD simulations (results for other two independent 25 ns simulations for each salt concentration are qualitatively similar and therefore not shown). (a) Overlap of IHF conformation in the IHF-DNA complex (PDB code 1IHF [8]) and a random snapshot by the end of the no-salt MD simulation. The backbone atoms of the core domain are used as the best-fitting region. The core and arm domains of the crystal conformation are shown in green and yellow, respectively, while those in the MD snapshot are shown in grey and purple, respectively. (b) and (c): the RMSD of backbone atoms of (b) the core domain and (c) the arm domain along MD trajectories with respect to the crystal conformation of IHF with the backbone atoms of the core domain as the best-fitting region.

Figure 3.

Normalized histogram $H(r)$ of salt bridge distance r_{min-NO} (the minimal distance between N in a cationic residue and O in all anionic residues) for all 36 cationic residues collected from no-salt and high-salt simulations. The inset shows the small peak between 3 and 6 Å.

Figure 4.

Representative behaviors of salt-bridges involving binding site cationic residues during MD simulations with no-salt (0 M KCl, top panel for each residue) and high-salt (350 mM KCl, bottom panel for each residue) conditions; results for other two independent 25 ns simulations for each salt concentration are qualitatively similar and therefore not shown. The salt-bridge distance r for a specific cationic residue is the minimal distance between its side-chain N atom and side-chain O atoms in all anionic residues; r_{init} indicates the value in the crystal structure. For the behaviors of other cationic residues (both in and beyond the DNA binding site), see Supporting Information.

Figure 5.

Behavior of cationic residues in IHF during MD simulations. (a) Correlation between $P_{no-salt}$ and P_{salt} for all 36 cationic residues. The 17 binding-site cationic residues are shown as red dots while the other 19 are shown as open circles; for each residue, results for three independent pairs of simulations are shown as separate data points. The circled red dots indicate four residues that exhibit the most significant differences between $P_{no-salt}$ and P_{salt} (also see Table 1). The line of $P_{no-salt} = P_{salt}$ is meant to guide the eyes. (b) A random snapshot from the no-salt simulation. IHF is shown in New Cartoon and colored according to the RMSF (red, high RMSF; blue, low RMSF). The 17 binding-site cationic residues are shown in licorice and in different colors according to their salt-bridge formation probabilities: residues whose $P_{no-salt}$ and P_{salt} are both greater than 0.10 are shown in red; residues whose $P_{no-salt}$ and P_{salt} are both lower than 0.10 are shown in blue; residues with high $P_{no-salt}$ but low P_{salt} (bold in Table 1 and circled in (a)) are shown in yellow.

Figure 6.

The positions of K3, K20, E23, D24, K27 on the β subunit in (a) the IHF conformation in the IHF-DNA complex (PDB code 1IHF [8]) and (b) a snapshot at 22.4 ns from the no-salt simulation. The five charged residues are shown in licorice scheme while the protein is shown in ribbons. The salt bridge distance (r_{min-NO} in Å) for salt bridges are also shown. Comparison between (a) and (b) indicates that the salt bridge pattern involving K20 β changes during the simulation.

Figure 7.

The differential residue-averaged-electrostatic-potential ($\Delta\phi_{res}$) between the simulated *apo* structure and the crystal *apo* structure, mapped onto the crystal *apo* structure. $\Delta\phi_{res}$ is larger than $5 \text{ kcal}\cdot\text{mol}^{-1}e^{-1}$, less than $-5 \text{ kcal}\cdot\text{mol}^{-1}e^{-1}$ and between -5 and $5 \text{ kcal}\cdot\text{mol}^{-1}e^{-1}$ for the blue, red and white regions, respectively.

Figure 8.

Results of DNA binding site prediction using two popular on-line servers; IHF is shown in New Cartoon scheme and the residues predicted the binding sites are shown in blue and vdW scheme. (a)–(b): The DNA binding sites predicted by PPF for the (a) crystal *apo* and (b) simulated unbound IHF structures. (c–d): The DNA binding sites predicted by Preds for (c) the crystal *apo* structure and (d) the simulated unbound structure.

Table 1

r_{init} , $P_{no-salt}$ and P_{salt} for the 17 binding-site cationic residues^a

Residue	r_{init} (Å)	$P_{no-salt}$	P_{salt}	Residue	r_{init} (Å)	$P_{no-salt}$	P_{salt}
K20α	3.87	0.99	0.95	K3β	7.38	0.10	0.12
		0.96/0.97	0.98/0.97			0.19/0.13	0.07/0.00
K24α	7.03	0.22	0.31	K27β	3.72	0.80	0.42
		0.05/0.14	0.16/0.00			0.86/0.59	0.53/0.61
K45α	7.58	0.00	0.00	R56β	12.39	0.28	0.00
		0.00/0.00	0.00/0.00			0.33/0.32	0.00/0.00
R55α	5.93	0.99	0.83	R59β	9.33	0.00	0.00
		0.89/0.83	0.63/0.03			0.00/0.02	0.00/0.00
K57α	10.00	0.00	0.00	K75β	5.22	0.60	0.89
		0.00/0.00	0.00/0.00			0.95/0.65	0.89/0.87
R60α	11.98	0.02	0.00	K81β	9.45	0.00	0.00
		0.00/0.00	0.00/0.00			0.00/0.00	0.00/0.00
R63α	4.40	0.42	0.68	R87β	5.91	0.90	0.00
		0.38/0.93	0.90/0.66			0.55/0.99	0.00/0.00
R76α	6.55	0.77	0.00				
		0.01/0.00	0.00/0.00				
R82α	4.41	0.63	0.70				
		0.57/0.60	0.70/0.75				
K88α	9.28	0.55	0.00				
		0.03/0.16	0.00/0.00				

^a r_{init} (in Å) is the minimal distance between N in a cationic residue and O in all anionic residues in IHF in the IHF-DNA complex (PDB code 1IHF [8]); $P_{no-salt/salt}$ is the probability of salt bridge formation in no-salt and high-salt simulations; residues shown in **bold** exhibit the most significant difference between $P_{no-salt}$ and P_{salt} (also shown as circled red dots in Fig. 5). For each residue, the first row indicate values from a 50 ns simulation, while the second row indicate values from two other independent 25 ns simulations.

Table 2

r_{init} , $P_{no-salt}$ and P_{salt} for the other 19 cationic residues^a

Residue	r_{init} (Å)	$P_{no-salt}$	P_{salt}	Residue	r_{init} (Å)	$P_{no-salt}$	P_{salt}
K5α	2.96	0.87	0.97	R9β	2.94	0.76	0.96
		0.99/0.96	1.00/0.99			0.97/0.97	0.94/0.91
K15α	4.56	0.86	0.92	K20β	2.97	0.03	0.06
		0.96/0.91	0.89/0.86			0.02/0.15	0.04/0.02
R21α	5.35	0.43	0.54	R42β	3.00	0.76	0.88
		0.66/0.43	0.58/0.31			0.70/0.52	0.32/0.24
R35α	2.87	1.00	1.00	R46β	2.96	0.28	0.34
		1.00/1.00	1.00/1.00			0.11/0.25	0.39/0.21
R36α	3.25	0.88	0.65	R62β	6.70	0.00	0.39
		0.79/0.79	1.00/0.81			0.00/0.00	0.00/0.00
K66α	12.39	0.08	0.00	K65β	10.08	0.00	0.00
		0.00/0.00	0.00/0.00			0.00/0.00	0.00/0.00
R77α	3.57	0.83	0.94	K69β	6.62	0.36	0.26
		0.81/0.58	0.89/0.96			0.32/0.51	0.14/0.20
K86α	7.24	0.02	0.05	K84β	2.95	0.70	0.31
		0.10/0.02	0.14/0.00			0.40/0.81	0.26/0.25
R90α	2.97	1.00	1.00	R89β	2.82	0.99	1.00
		0.98/0.99	1.00/1.00			0.95/1.00	1.00/1.00
K97α	4.52	0.55	0.56				
		0.46/0.57	0.56/0.42				

^aSee footnote of Table 1 for notation.