Rigid-body motions of interacting proteins dominate multispecific binding of ubiquitin in a shape-dependent manner

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ABSTRACT

To understand the dynamic aspects of multispecificity of ubiquitin, we studied nine ubiquitin–ligand (partner protein) complexes by normal mode analysis based on an elastic network model. The coupling between ubiquitin and ligand motions was analyzed by decomposing it into rigid-body (external) and vibrational (internal) motions of each subunit. We observed that in total the external motions in one of the subunits largely dominated the coupling. The combination of external motions of ubiquitin and the ligands showed general trends of rotations and translations. Moreover, we observed that the rotational motions of ubiquitin were correlated to the ligand orientations. We also identified ubiquitin atomic vibrations that differentiated the orientation of the ligand molecule. We observed that the extents of coupling were correlated to the shapes of the ligands, and this trend was more pronounced when the coupling involved vibrational motions of the ligand. In conclusion, an intricate interplay between internal and external motions of ubiquitin and the ligands help understand the dynamics of multispecificity, which is mostly guided by the shapes of the ligands and the complex.

INTRODUCTION

Protein–protein recognition is a process in which two proteins specifically interact in a cellular environment.1 Such a specificity of interaction is tightly regulated in a cell. Hub proteins are a type of proteins that show promiscuous binding (i.e., multispecific binding) to many partner proteins (i.e., ligands) synchronously (in case of party hub proteins) or asynchronously (in case of date hub proteins).2 Previously, we studied how the multispecificity of date hub proteins can be characterized from the promiscuity of atomic sites without considering any dynamic aspect.3 The present definition of multispecificity includes selectivity (i.e., how ubiquitin selects its ligand proteins out of the nonligands) and specificity (i.e., how at a given time only one of all other ligands bind ubiquitin). A growing number of studies indicate dynamics play a key role in the protein–protein recognition.4–7 In a previous study,4 to indicate that the global dynamics may help in protein–protein recognition Bai et al. discriminates biological and nonbiological complexes by comparing residue fluctuations. Such a study confirms that ligands and nonligands show different dynamic characteristics. Here, we characterized major dynamic aspects that are directly correlated to the multispecificity of a date hub protein ubiquitin.

It has been previously observed that many date hub proteins use overlapping (OV) regions of interfaces to bind its ligands asynchronously.8,9 However, at a given moment a protein–protein interface includes a specific set of atoms in addition to the OV region. Previously, we defined a multiligand interface that integrates all the interfaces on a date hub protein, and we also defined “overlapping” or “OV” and “nonoverlapping” or “non-OV” regions in the multiligand interface based on the promiscuity of the interface sites.3 In a recent study, we observed that ligand-coupled vibrational dynamics at the OV and non-OV regions dictate conformational change due to binding, and therefore correlated to the
The dynamic aspects of a protein–protein interaction could be obtained from an all-atom normal mode analysis (AA-NMA) of an elastic network model (ENM).\[^{5,12,13}\] For a dimeric protein complex ("RL," where "R" and "L" are the receptor and ligand subunits) consisting of \( N \) atoms one obtains \( 3N - 6 \) vibrational modes from AA-NMA. These \( 3N - 6 \) modes include \( 3N^R - 6 \) vibrational modes obtained from the receptor subunit "R" of \( N^R \) atoms, \( 3N^L - 6 \) vibrational modes obtained from the ligand subunit "L" of \( N^L \) atoms, and six modes characterizing the relative rigid-body motions of the subunit "R" with respect to the subunit "L."\[^{10}\] The coupling of \( 3N^R - 6 \) motions of the receptor to the \( 3N^L - 6 \) motions of the ligand and six relative rigid-body motions are embedded in a rectangular submatrix of the covariance matrix yielded by the AA-NMA. From the rectangular matrix, it is possible to separate rigid-body and vibrational motions of the subunits by defining suitable projection matrices (schematically shown in Fig. 1). Here, the motions of receptor that are coupled to the motions of the ligand are referred as "ligand-coupled motions".

In the present analysis, ubiquitin is taken as a model date hub protein, because of its highest promiscuity among other date hub proteins\[^{3}\] obtained from the structures in Protein Data Bank (PDB).\[^{14}\] Ubiquitin is a \( \beta \)-grasp folded protein,\[^{15}\] sequence of which is well conserved in eukaryotes.\[^{16}\] Ubiquitin not only labels hijacks host machinery by targeting ubiquitin (Table 1).\[^{17,18}\] Therefore, understanding how ubiquitin recognizes its multiple ligands is of immense biological importance and this is the goal of the present analysis.

**MATERIALS AND METHODS**

**Data set of ubiquitin heterodimers**

The heterodimer data set that we used here is the same as in our recent study.\[^{10}\] There are nine representative complexes (Table I; Supporting Information Fig. S1), obtained by refining a set of 87 protein–protein interfaces (obtained in our earlier study\[^{3}\] that includes nonrepresentative structures). All the nine heterodimers are annotated as biological in PDB and solved by X-ray crystallography.\[^{19}\] The ligands in the data set are diverse with respect to their sequences (maximum sequence similarity\[^{20}\] is 31% with e-value \( 1 \times 10^{-8} \) between the ligands in the complexes "1WRD" and "1WR6"). The ubiquitin chains in some complexes do not include the last four residues. Therefore, for consistency the sequence of ubiquitin was truncated to 72 residues (i.e., the last four residues ignored). We superimposed\[^{21}\] ubiquitin chains of nine heterodimers to a monomeric ubiquitin structure (1UBQ, residues 1–72).

**Definitions of multiligand interface and OV and non-OV regions**

The multiligand interface was defined from the integration of the ubiquitin interface residues in the initial
Table 1
Data Set of Ubiquitin-Associated Biologically Relevant Heterodimers (Used in Current Study) That Share Overlapping Interfaces

| PDBID | Receptor | Ligand | Ligand annotation | Shape of the ligand (asphericity) |
|-------|----------|--------|-------------------|----------------------------------|
| 2J7Q  | D        | C      | MCMV<sup>α</sup> tegument protein M48-encoded ubiquitin- specific protease, M48<sub>SP</sub> | 0.005 |
| 2H0H  | A        | B      | Vacuolar protein sorting protein 36 | 0.006 |
| 2O0B  | G        | H      | E3 ubiquitin-protein ligase EDD1 | 0.013 |
| 151Q  | D        | C      | E3 ubiquitin-protein ligase CBL-B | 0.020 |
| 3C0R  | H        | D      | Tumor susceptibility gene 101 protein | 0.026 |
| 1WR6  | B        | A      | Ubiquitin thiosterase OTU1 | 0.046 |
| 1WRD  | B        | A      | ADP-ribosylation factor binding protein GGA3 | 0.096 |
| 2C7M  | B        | A      | RAB guanine nucleotide exchange factor 1 | 0.108 |

<sup>a</sup>Chain identifiers were defined from the label asymmetric identifiers given in the corresponding PDBML files.<sup>19</sup>

<sup>b</sup>The rows are sorted in increasing order of the asphericity. Asphericity (\(\lambda\))<sup>27</sup> is a measure of departure from the spherical shape of the ligands and defined as, \(\Lambda = \frac{\sum_{i=1}^{N} (\lambda_i - \lambda_N)^2}{\sum_{i=1}^{N} \lambda_i} \), where \(\lambda_i\) is the moment of inertia and \(\lambda_N\) is the moment of inertia tensor, \(\Lambda\) is bounded between 0 and 1, where 0 indicates spherical shape and 1 indicates rod shape.

<sup>c</sup>The source organism of the ligand (obtained from the PDBML file) is a virus, that hijacks the host machinery by its deubiquitinating activity.<sup>17</sup>

<sup>d</sup>“MCMV” stands for murine cytomegalovirus.

A set of 87 interfaces. The definitions of the OV and non-OV regions were adopted from our recent study.<sup>10</sup> There are 22 and 36 residues (178 and 286 atoms, respectively) in the OV and non-OV regions [Supporting Information Fig. S2(a)], respectively. The remaining 14 residues (110 atoms) are solvent exposed.<sup>22</sup>

**AA-NMA of the ubiquitin complexes**

We performed AA-NMA of nine ubiquitin complexes (Supporting Information Fig. S1) based on an ENM<sup>12</sup> using our in-house program.<sup>10</sup> In ENM a protein structure is modeled as a set of Hookean springs, where distance between any two atoms is restrained to its native value. The potential energy \((V)\) of such a system is given by,

\[
V = \frac{1}{2} \sum_{i,j} K_{ij} (d_{ij} - d_{ij}^0)^2
\]  

(1)

where \(d_{ij}\) is the distance between atoms \(i\) and \(j\), and superscript “0” indicates native value (assumed to be crystallographic condition in ENM). \(K_{ij}\) is the force constant of the spring connecting atoms \(i\) and \(j\), and we defined \(K_{ij} = \exp[(1/2) (d_{ij}^0 / d_{cutoff})^2]\), where \(d_{cutoff}\) is 5 Å.

For a system of \(N\) atoms, there will be \(3N\) degrees of freedom, and \(V\) is a function thereof. These \(3N\) degrees of freedom are obtained from the Cartesian coordinates \((x, y, z)\) of each atom \((q_{ix}, q_{iy}, q_{iz})\) expressed as \(q_{iα}\), where \(α \in \{x, y, z\}\).

The \(3N \times 3N\) Hessian matrix \((\hat{H})\) of the above potential function \(V\) [Eq. (1)] can be obtained analytically,

\[
\hat{H}_{\alpha\beta} = \frac{\hat{\alpha}^2 V}{\hat{q}_{\alpha} \hat{q}_{\beta}} = K_{ij} \frac{\Delta q_{\alpha}^0 \Delta q_{\beta}^0}{(d_{ij}^0)^2}, \quad i \neq j
\]

(2)

where \(\Delta q_{\alpha}^0 = q_{\alpha}^0 - q_{\alpha}^0\), \(\Delta q_{\beta}^0 = q_{\beta}^0 - q_{\beta}^0\), and \(\alpha, \beta \in \{x, y, z\}\).

The displacements of atom \(i\) along one of its generalized coordinate \((\alpha)\) is given as,

\[
q_{\alpha} - q_{\alpha}^0 = \Delta q_{\alpha} = \frac{1}{\sqrt{m_i}} \sum_{k=1}^{3N-6} \kappa_k a_{i\alpha, k} \cos(\lambda_k^{1/2} t + \phi_k) \tag{3}
\]

where \(m_i\) is mass of atom \(i\), \(a_{i\alpha, k}\) is mass-weighted component of \(k\)-th eigenvector for atom \(i\) and coordinate \(\alpha\), \(\phi_k\) is phase of \(k\)-th eigenvector, and \(\kappa_k\) = \((2K_B T)^{1/2}/\lambda_k^{1/2}\) by assuming the principle of equipartition of energy, where, \(K_B\) is the Boltzmann constant and \(T\) is absolute temperature (taken to be 300 K). The covariance matrix \((C)\) that was computed from Eq. (3) is given by

\[
C_{\alpha\beta} = \langle (q_{\alpha} - q_{\alpha}^0)(q_{\beta} - q_{\beta}^0) \rangle = \frac{K_B T}{\sqrt{m_i m_j}} \sum_{k=1}^{3N-6} a_{i\alpha, k} a_{j\beta, k} \lambda_k \tag{4}
\]

where \(\Lambda_k\) is diagonal matrix containing the \((3N - 6)\) nonzero eigenvalues of \(\hat{H}\) (superscript “T” indicates transpose operation).
The covariance matrix given in Eq. (4) is a $3N \times 3N$ matrix, which was further split into $M_{RL}$ and $M_{LL}$ square submatrices, $M_{RL}$ and $M_{LR}$ rectangular submatrices ("R" represents receptor and "L" represents ligand, Fig. 1). The $M_{RL}$ submatrices were analyzed in detail in this study.

**Separation of external and internal motion from the total motion of ubiquitin or ligands**

The covariance matrix $C$ obtained from Eq. (4) indicates how ubiquitin receptor and the corresponding ligand are fluctuating keeping the whole complex in the Eckart frame. In this case, the fluctuations of the receptor and ligand were due to the total motions of the receptor and ligand, respectively. Such total motion of a subunit is composed of internal vibrational motion of that subunit and external rotational, translational movement of one subunit with respect to the other.

The $3N^R - 6$ and $3N^L - 6$ vibrational modes from the receptor and ligand subunits are defined as the internal modes of the receptor and ligand, respectively. We are primarily interested in the relative external motion of the receptor with respect to the ligand. The relative motions of the receptor with respect to the ligand are embedded in the covariance matrix that includes receptor–ligand coupled motions [the rectangular part of the covariance matrix $M_{RL}$ given in Eq. (4)]. The relative external motion of the receptor and ligand can be obtained by transforming such a covariance matrix to put both the receptor and ligand in their non-Eckart frame simultaneously (Fig. 1). For the above transformations projection matrices for the receptor and ligand parts are defined from their atomic coordinates. For the receptor part the projection matrix is defined as,

$$P^R = 1 - (t_x t_x^T + t_y t_y^T + t_z t_z^T + r_x r_x^T + r_y r_y^T + r_z r_z^T)$$  

where $t_x$, $t_y$, and $t_z$ are translational vectors [e.g., $t_x$ elements are normalized set of $M^{1/2}(1, 0, 0, \ldots)^T$, $t_y$ elements are normalized set of $M^{1/2}(0, 1, 0, \ldots)^T$, and $t_z$ elements are normalized set of $M^{1/2}(0, 0, 1, \ldots)^T$, where $M$ is mass matrix] and $r_x$, $r_y$, and $r_z$ are rotational vectors (e.g., $r_x$ elements are normalized set of $M^{1/2}(0, -z_1, y_1, \ldots)$, $r_y$ elements are normalized set of $M^{1/2}(z_1, 0, -x_1, \ldots)$, $r_z$ elements are normalized set of $M^{1/2}(-y_1, x_1, 0, \ldots)$, where $x_1$, $y_1$, and $z_1$ are coordinates of atom 1) and "1" in Eq. (5) is the identity matrix. It is important to note that such formulations of translational and rotational vectors is only applicable when the rigid-body movements are infinitesimally small; otherwise the rotational vectors will not be orthogonal to each other. This can be solved by orthonormalizing the set of six translational and rotational vectors, which will decouple the interdependence of all rotations.

In the present study, we asked how the internal and external motions of receptor were coupled to the internal and external motions of the ligand. The rectangular submatrix $M_{RL}$ of $C$ (Fig. 1) was used to obtain different combinations of internal and external coupling between the ligand and are obtained by,

$$M_{RL}^{(i-j)} = p^R M_{RL}^{(i-j)} p^L$$ (6a)
$$M_{RL}^{(c-e)} = (1 - p^R) M_{RL}^{(i-j)} (1 - p^L)$$ (6b)
$$M_{RL}^{(i-c)} = p^R M_{RL}^{(i-j)} (1 - p^L)$$ (6c)
$$M_{RL}^{(i-n)} = (1 - p^R) M_{RL}^{(i-j)} p^L$$ (6d)

where $p^L$ is the projection matrix for the ligand.

To obtain coupling modes between internal, external, and total motions of the receptor and ligand, we performed singular value decomposition (SVD) of the matrices given by Eq. 6 and obtained singular values and corresponding left and right singular vectors for each combination of motions. For example, SVD of internal–external coupling of motion is given by,

$$M_{RL}^{(i-c)} = A_R^{(i-c)} S^{(i-c)} (A_L^{(i-c)})^T$$ (7)

where $A_R^{(i-c)}$ and $A_L^{(i-c)}$ are the left and right singular vectors corresponding to the receptor and ligand due to internal–external coupling of motion and $S^{(i-c)}$ contains corresponding singular values. In general, we write receptor $k$-th coupling modes as $A_R^{(m-n)}$, where "m" type of motion of the receptor is coupled to "n" type of motion of the ligand. Similarly, we write ligand $k$-th coupling modes as $A_L^{(m-n)}$ and $k$-th singular value as $S_k^{(m-n)}$. The coupled modes of the receptor and ligand were designated as holomodes in the present study. The components of the singular vectors can be interpreted as initial atomic velocities.

These SVDs were performed for all types of covariance matrices arising from nine complexes.

**Magnitude of initial velocities in different regions**

The initial velocity of atom $i$ (in three-dimensional space) due to mode $k$ in complex $c$ ($\vec{v}_{ik}^{(c)}$) was defined from the corresponding singular vector components obtained from the $M_{RL}$ covariance matrices. The magnitude of the corresponding initial velocity was defined as $||\vec{v}_{ik}^{(c)}||$. When this is averaged over atoms in the OV and non-OV regions, we obtained regionwise averaged magnitudes of initial velocities due to mode $k$ in complex $c$ ($||\vec{v}_{ik}^{(c)}||_{OV}$ and $||\vec{v}_{ik}^{(c)}||_{Non-OV}$). For all complexes, we observed that in general $||\vec{v}_{ik}^{(c)}||_{OV}$ is greater than $||\vec{v}_{ik}^{(c)}||_{Non-OV}$ for low-frequency modes. Therefore, we averaged $||\vec{v}_{ik}^{(c)}||_{OV}$ and $||\vec{v}_{ik}^{(c)}||_{Non-OV}$ over all complexes.
and defined the complex-averaged magnitudes of initial velocities in OV and non-OV regions due to mode $k$ or $h_{k}||C_{22} v_{k}||_{i}^{OV}$ and $h_{k}||C_{22} v_{k}||_{i}^{Non-OV}$.

Similarly, we have defined complex-averaged magnitudes of initial velocities in the multiligand interface ($h_{k}||C_{22} v_{k}||_{Interface}$) and surface ($h_{k}||C_{22} v_{k}||_{Surface}$) using the atom sites in the multiligand interface and surface, respectively.

**RESULTS**

**Dominance of external motions in ligand-coupled motions of ubiquitin**

It was previously revealed that in the case of subtilisin–eglin C protein–protein complex, the external–external coupling largely influenced the total coupling of the subunits.\textsuperscript{11} We found that this also holds true for ubiquitin when the coupling of six lowest frequency motions between receptor and ligands were considered. To show this, we have measured the contributions of different types of partial couplings to the total coupling [Fig. 2(a)]. We observed that each of the total coupling modes was contributed by only a few partial coupling modes among which the ones involving external motions of the receptor were prominent [Fig. 2(b)]. The internal–external coupling prominently contributes to the total coupling after the first six modes up to around the 15th mode. The total coupling modes of even higher orders were predominantly contributed by internal–internal coupling.

**Combination of rigid-body motions correspond to orientations of ligands**

To characterize the dynamics of the external–external couplings in nine complexes, we visually inspected dynamics from the corresponding singular vectors. These singular vectors were obtained from the SVD of the covariance matrix and are referred to as rigid-body modes (Supporting Information Fig. S3). The six external–external coupling modes describe the couplings between rigid-body motions of the receptor and a ligand. The nonzero angular momentum and linear momentum in every receptor external mode (Fig. 3) indicate that the rotational and translational motions are mixed. Nevertheless, we observed that the receptor motions showed higher angular momentum and lower linear momentum in the first three modes than in the next three modes, which indicates that the first three modes were similar to the rotational, and the next three modes were similar to the translational motions. However, for rod-shaped ligands the above trends were less clear due to greater mixing of the rotations and translations. From visual inspections, we conclude that the motions of receptor
and ligand are similar to the vibration of two rigid bodies in the fourth mode and they are similar to the shearing motions between the subunits in the fifth and sixth modes (Supporting Information Fig. S3). When different ligands were considered, we observed that the rotational axes change for the first three modes depending on the orientation of the ligand molecule (Fig. 4). The change in orientations of ubiquitin in response to the ligands is observed from the angle between its first principal moment of inertia in unbound state and its rotational axis obtained from the first external–external coupling of motion in nine ligand-bound states [Fig. 5(a)]. Such angles range from $8^\circ$ to $72^\circ$ exhibiting a large change in ubiquitin orientation in different ligand environment.

**Rotation of ubiquitin is aligned to the principal axis of inertia**

We also observed that in each of the first three external–external coupling modes in each complex the rotational axis of the receptor is nearly parallel to that of the ligand (average angle $10.09 \pm 7.38^\circ$). This observation prompted us to investigate how ubiquitin external motion is influenced by the presence of a ligand. We compared three principal axes of inertia of ubiquitin to the rotational axes of the receptor motions observed in the first three modes of external–external couplings [Fig. 5(b)]. We observed that the rotational axis from the first mode was more aligned to the first principal axis of inertia and the rotational axis from the third mode was more aligned to the third principal axis of inertia. This can be explained from the fact that the first external–external coupling mode is associated to the slowest motion and is aligned to the axis of the largest inertia. Although rotational axes change depending on the different ligand orientations [Figs. 4 and 5(a)], on average, the rotational motions of bound ubiquitin were largely influenced by the intrinsic rotational properties of ubiquitin.

**Combinations of vibrational and rigid-body motions show shape specificity**

To understand how the shape of the ligand (Table I, last column and Supporting Information Fig. S1) affects the coupled dynamics, we measured the correlation coefficient between the singular values in the nine complexes for the first six coupled motions (Supporting Information Table S2) and the asphericity of the ligands $^27$ (Table II). We observed that the first, second and fifth singular values obtained from the total coupling were correlated to the asphericity of the ligand molecules: more rod-shaped ligands had greater magnitudes of coupling of motion (Table II). When internal motions of ligand molecules were involved in the partial coupling (viz. internal–internal and external–internal couplings) strong correlations between the nonglobular nature of the ligand and the magnitude of coupling were observed. For
the external–external coupling of motion only the translational modes (fifth and sixth modes) were correlated with the shape of the ligand. For internal–external coupling all the correlation coefficients were found to be insignificant. Therefore, in general, the correlation between the ligand shapes to the magnitudes of coupling was underscored when internal motion of ligand was coupled to the receptor motions. Qualitatively, the internal motion of a subunit is correlated with its shape more strongly than the external motion of the subunit. In summary, the extent of coupling (except internal–external coupling) was higher for rod-shaped ligands than for spherical ligands.

**Figure 4**
Change in the axes of rotation in two complexes (in stick representation). (a) Difference in orientations of the ligands from the complexes 1WRD and 2C7M (in green and red, respectively). Ubiquitin chain is shown in blue. (b) first external–external coupling modes from the above complexes. The directions of atomic motions from ubiquitin and the ligands are shown in magenta and orange, respectively.

**Atomic vibrations of ubiquitin related to the rigid-body motions of the ligands**

In previous sections, we observed that the shape and orientation of the ligands are correlated to the external–internal and external–external couplings. Next, to understand how the vibrational motions of ubiquitin recognize rigid-body motions of the ligands, the left singular vectors of internal–external covariance matrices obtained from the ligand-coupled motions of ubiquitin in different complexes were analyzed. In particular, we analyzed internal motions of atoms that are fluctuating significantly.

We identified which atoms and residues of ubiquitin are exhibiting high displacements due to the internal–
external coupling (Table III). Table III shows that side-chain atoms exhibit high displacement in many cases. The high atomic displacements in the N-terminal β-hairpin region and in the β-hairpin (including Ala46 and Gly47) preceded by functionally important Ile44 residue\(^28\) of ubiquitin were prominent. Moreover, we observed that polar atoms of the side chains in Asp39, Arg42, and Lys63 residues are highly flexible. For multispecific proteins, it is already known that strong electrostatic interaction between charged residues help to explain diverse binding poses.\(^29–32\) Our result shows that even without using charge interactions explicitly (as in ENM) polar side chains may adjust to different ligands. The flexibility of side chain motions in those residues may help ubiquitin to acquire multiscifcity.

We observed that the directions of atomic motions (obtained from the lowest frequency internal–external ubiquitin modes in three complexes) at the N-terminal β-hairpin region [loop “L1” in Supporting Information Fig. S2(b)] change depending on the orientations and positions of different ligands (Fig. 6). Due to the change in ligand binding modes the overall shapes of the complexes change (Supporting Information Fig. S1). Of the three complexes shown in Figure 6, the overall shape of the ubiquitin-ADP-riboseylation factor binding protein GGA3 complex is similar to that of the ubiquitin target of Myb protein 1 [Fig. 6(a)], but different from that of the ubiquitin-E3 ubiquitin–protein ligase CBL-B [Fig. 6(b)]. The orientations of the ligands shown in Figure 6(a) are slightly different because one is more tilted with respect to the other. From Figure 6(b), it is evident that the binding mode of E3 ubiquitin–protein ligase CBL-B [Fig. 6(b)] is quite different from the other two. We observed that in general the collective motions of the atoms are similar to a rotation of the loop slipping away from the ligand and therefore minimizing collision between the subunits. Apparently, directions of motion of the loop residues are similar when orientations of the ligands were similar [Fig. 6(c,d)], and different when orientations of the ligands were different [Fig. 6(c–e)]. However, a closer inspection reveals subtle changes [Fig. 6(f)]. For example, considering the motions of Lys6 side-chain amide nitrogen atom in three complexes, we observed that the angle between the atomic displacement vectors is largest (25°) when motions from 1WRD and 2OOB complexes are considered. The average angle obtained from the other two pair of complexes, (i.e., 1WR6, 2OOB pair, and 1WR6, 1WRD pair) is 14°.

### Table II

| Coupling of motion | Order of holo mode |
|--------------------|--------------------|
| Receptor–ligand    | 1 2 3 4 5 6        |
| Total–total        | 0.86 0.73 0.56 0.64 0.72 0.60 |
| External–external  | 0.59 0.62 0.25 0.59 0.85 0.74 |
| Internal–external  | 0.24 0.30 0.00 0.38 0.47 0.30 |
| External–internal  | 0.87 0.84 0.81 0.70 0.75 0.58 |
| Internal–internal  | 0.81 0.71 0.72 0.77 0.71 0.69 |

*The correlation indicates Pearson correlation coefficient between the singular values of holo modes of same order from nine complexes (Supporting Information Table S1) and shape parameters of corresponding ligands (Table I). The values in italics represent insignificant correlations.
The Lys6 of ubiquitin is considered to be a high affinity binding site and was observed to show considerable change in the ligand-induced chemical shifts in NMR experiments. It was postulated that the highly polar nature of this residue gives rise to strong electrostatic interactions and helps to show multispecificity. The significant flexibility of this residue observed in the current study may help ubiquitin to recognize different ligands. Moreover, from our previous study, we recall that the internal–external coupled motions are embedded in the motions of unbound ubiquitin.

In conclusion, side-chain flexibility and change in the N-terminal loop motions of ubiquitin may help it to recognize ligand orientations through internal–external coupling of motions.

**DISCUSSION**

Rigid-body motions of one subunit relative to the other are nothing but the vibrational motions that are included in the motions of the complex. The coupled rigid-body motion was shown to be crucial to stabilize the bound form of a permanent complex, as observed by Ishida et al. In this analysis of subtilisin–eglin C complex, Ishida et al. found large negative correlations between internal and external motions within each of the constituent molecule. Such correlations alleviate unfavorable entropy loss due to suppression of atomic fluctuations at the binding regions. Moreover, they indicated that a large positive correlation between the external motions of subtilisin and eglin C helps to stabilize the

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**Table III**

| Ubiquitin residues | 2J7Q | 2H7H | 2Q9H | 2O0B | 1SIQ | 3COR | 1WR6 | 1WRD | 2C7M |
|-------------------|------|------|------|------|------|------|------|------|------|
| Lys6              | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   |
| Thr7              | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   |
| Leu8              | MC, SC | MC, SC | MC, SC | SC  | SC  | MC, SC | MC, SC | MC, SC | MC, SC |
| Thr9              | MC, SC | MC, SC | MC, SC | MC   | MC   | MC, SC | MC, SC | MC, SC | MC, SC |
| Gly10             | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   |
| Lys11             | MC, SC | MC, SC | MC, SC | SC  | SC  | MC, SC | MC, SC | MC   | MC   |
| Thr12             | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   |
| Gin31             | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   |
| Gin35             | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   |
| Ile36             | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   |
| Pro37             | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   |
| Pro38             | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   |
| Asp39             | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   |
| Glu40             | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   |
| Arg42             | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   |
| Ile44             | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   |
| Phe45             | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   |
| Ala46             | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   |
| Gly47             | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   |
| Lys48             | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   |
| Gin49             | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   |
| Glu51             | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   |
| Arg54             | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   |
| Ser57             | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   |
| Tyr59             | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   |
| Asn60             | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   |
| Gin62             | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   |
| Lys63             | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   |
| Glu64             | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   |
| Ser65             | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   |
| His68             | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   |
| Val70             | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   | SC   |
| Leu71             | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   |
| Arg72             | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   | MC   |

*Residues in italics are in non-OV region. Otherwise, they are in OV region.*

*For a complex "MC" or "SC" annotation indicates at least one of the main-chain or side-chain atoms from the residue showed significantly high mean square fluctuation from the six modes. The atom-wise mean square fluctuations are reported in Supporting Information Table S2. A "-" indicates that the atoms of the corresponding residue do not show high fluctuation in a complex.*

*The consensus annotation is based on the annotations from nine complexes. For Gin62 in only one complex (from PDB identifier 1SIQ) main-chain carbonyl carbon was showing high fluctuation (Supporting Information Table S2), and this was ignored in its consensus annotation.*

The Lys6 of ubiquitin is considered to be a high affinity binding site and was observed to show considerable change in the ligand-induced chemical shifts in NMR experiments. It was postulated that the highly polar nature of this residue gives rise to strong electrostatic interactions and helps to show multispecificity. The significant flexibility of this residue observed in the current study may help ubiquitin to recognize different ligands. Moreover, from our previous study, we recall that the internal–external coupled motions are embedded in the motions of unbound ubiquitin.

In conclusion, side-chain flexibility and change in the N-terminal loop motions of ubiquitin may help it to recognize ligand orientations through internal–external coupling of motions.
bound form. This confirms the importance of external motions in the formation of stable complexes.

In our recent study, we observed that coupled rigid-body motion optimized complementarity of motions at the interface. In the present study, we observed that coupled rigid-body motions dominated the most collective total coupling motions (Fig. 2). To compare the collective behaviors of different types of partially coupled
motions, we compared their cumulative singular values (Fig. 7) that are obtained from the SVD of the covariance matrices. For the first few modes, we observed that the trends obtained from the different types of coupled external motions match well to the total coupling of motion. This indicates a dominance of external motions for the first few modes. Moreover, the motions that involved external motions either in the receptor or ligand are slower than the internal–internal coupling. It has been shown for adenylate kinase\textsuperscript{34} and \textit{Escherichia coli} CheY\textsuperscript{35} that co-occurrence of slow and fast dynamics are slower than the internal–internal coupling. It has involved external motions either in the receptor or ligand motion. This indicates a dominance of external motions. For the first few modes, we observed that (Fig. 7) that are obtained from the SVD of the covariance matrices. For the first few modes, we observed that the trends obtained from the different types of coupled external motions match well to the total coupling of motion. This indicates a dominance of external motions for the first few modes. Moreover, the motions that involved external motions either in the receptor or ligand are slower than the internal–internal coupling. It has been shown for adenylate kinase\textsuperscript{34} and \textit{Escherichia coli} CheY\textsuperscript{35} that co-occurrence of slow and fast dynamics are related to their functions. From our current and previous\textsuperscript{10} studies, we observed that faster internal–internal coupling of motion and relatively slower coupled external motions (e.g., external–external coupling of motions) are correlated to multispecificity.

The rigid-body motions of ubiquitin or ligands involved its rotational and translational motions. In the present method, it is not possible to separate rotational and translational motions from each other (Fig. 3), due to mixing of those motions while defining a projection matrix used for decoupling internal and external motions. However, we observed more rotation-like motions in the first three modes of external–external coupling (prominent for spherical ligands), demonstrating the presence of relative rotational motions of the two subunits slower than the relative translational motions. For such slow motions the principal axis of inertia of ubiquitin was mostly aligned to its rotational axes (Fig. 5). This indicates that in a longer timescale the rotation of ubiquitin is primarily guided by its intrinsic rotational properties even in the environment of ligands, thereby recognizing multiple ligands similarly and being able to select them all. However, at a given moment ubiquitin will bind specifically to one of the ligands. To explain this on the basis of external–external coupling, we observed slight changes in rotational axes of ubiquitin due to different orientations of the ligands (Fig. 4).

The external–external coupling is related to how the ligand molecule is oriented with respect to the receptor or overall quaternary structure of the heterodimer. Depending on the orientation of the ligands a specific set of receptor atoms will be involved in the ligand binding.\textsuperscript{36} For a protein–protein complex the dynamic encounter complexes formed before the formation of the final bound state involve long-range electrostatic interactions and multiple relative orientations of interacting subunits.\textsuperscript{31} In addition, other studies showed that long-range electrostatic interactions enhance ligand specificity and affect promiscuity.\textsuperscript{29,30} Patil et al.\textsuperscript{32} showed that for small hub proteins such as ubiquitin, enrichment of surface charged residues is related to its multispecificity. How a protein optimizes its final binding mode from such multiple relative orientations and a specific set of interacting residues? The previous study of Patil et al.\textsuperscript{33} indicated that the set of interacting residues in a final protein complex involve short-range hydrophobic interactions and hydrogen bonds across the interface that optimizes the final bound form. In the current study, we observed that on an average the rotational axes from the first rigid-body mode of ubiquitin and ligands are nearly parallel. However, the rotation-like rigid-body motions of ubiquitin in bound forms match well to its rotational motion in the unbound form [Fig. 5(b)]. It might be possible that such characteristics of rigid-body motions may help ubiquitin to acquire the correct binding pose.

One of the important aspects of this study is the correlation of vibrational motions of a subunit to its globular or rod-shaped nature (Table II). The vibrations of a rod-shaped ligand are strongly coupled to the motions of ubiquitin. This implies that the ligand shape may guide the specific binding to a ligand (most prominent from the external–internal coupled motion, Table II). Therefore, the multispecificity of ubiquitin also depends on the intrinsic properties of the ligands. A similar observation has been made in the analysis of multiple binding to Calmodulin by Fromer and Shifman.\textsuperscript{37} In our previous analysis, we observed that the external–internal and external–external ligand-coupled motions of ubiquitin were not embedded in the vibrational motions of apo ubiquitin.\textsuperscript{10} Moreover, Kundrotas et al.\textsuperscript{38} recently showed that the protein–protein binding modes could be modeled from the homology modeling of the individual subunits. This indicates that the binding mode may be dictated in terms of the overall shape or fold of the

![Figure 7](image_url)

**Figure 7**  
Cumulative sum of singular values averaged over nine complexes for the first 30 modes yielded by total couplings and partial couplings. The cumulative sums are normalized by sum of all singular values. The “R(m)–L(n)” types of couplings in the legend indicates the coupling of the “m” type of motion of the receptor (“R”) to the “n” type of motion of the ligands (“L”).
subunits. This is also consistent with the present result of ligand-shape-dependent multispecificity of ubiquitin.

The partial couplings that we have analyzed here also include internal–external coupling, and internal motions of ubiquitin showed trends that were consistent with our earlier study (Supporting Information Fig. S4). For example, we observed that the flexibility in the OV region of ubiquitin is higher than the non-OV region. This indicates that flexibility and promiscuity of residue sites in the multiligand interface are correlated even in the case of the internal–external coupling. We already indicated that internal–external coupling modes are slower than internal–internal coupling modes (Fig. 7). Therefore, correlation between the flexibility and the promiscuity is also observed in longer timescale. This is consistent with an earlier NMR residual dipolar coupling experiment that showed similar correlation in millisecond timescale.

CONCLUSIONS

The dynamic aspects of multispecificity of ubiquitin were analyzed by decoupling of external and internal motions from the ligand-coupled motions. It was shown that the external motions of the interacting subunits dominated the ligand-coupled motion. We observed that the external motions of ubiquitin correlated well with the shape of the ligand and the complex in longer timescale. We have shown that due to change in orientations of the ligands the rotational axes of ubiquitin also change. However, on average such rotations follow rigid-body dynamics of unbound ubiquitin. Moreover, we showed that the atomic vibrations of ubiquitin might help recognize different orientations of the ligands. We emphasize that the current study mainly aims to answer how the dynamics and multispecificity are correlated. Nevertheless, we obtained a few dynamic characteristics that may contribute to the multispecificity. In summary, overall shapes of the interacting subunits were shown to be a major dynamic determinant of ligand specificity of ubiquitin.

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