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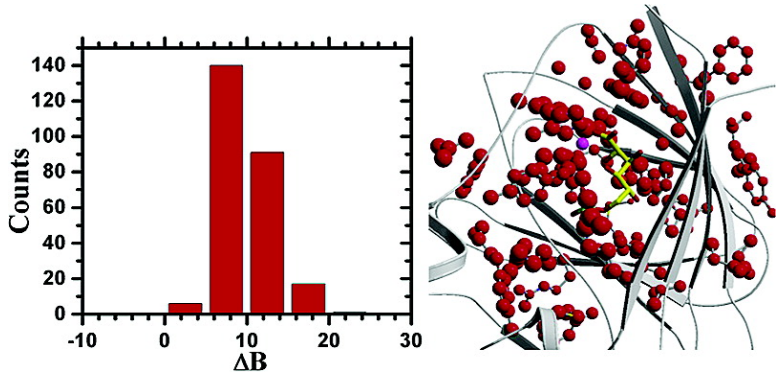

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A Systematic Analysis of the Effect of Small-Molecule Binding on Protein Flexibility of the Ligand-Binding Sites

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Abstract: Using atomic *B*-factors from high-resolution crystal structures of proteins and protein−ligand complexes, we have studied the effect of small-molecule ligand binding on protein flexibility. For the majority of proteins studied, ligand binding to a protein results in an increase in atomic mobility for some protein atoms at the ligand-binding site, and it leads to an increase in protein flexibility of the entire ligand-binding site in several cases.

Binding of small-molecule ligands to proteins is an important biochemical and biological process and is used as a basis for drug design. Such binding can cause large-scale conformational changes in proteins, and such changes have been observed in many experimentally determined three-dimensional structures. Binding also induces more subtle changes in proteins, including changes in atomic mobility and low-frequency vibrational motion.[1,2] Such subtle changes have entropic consequences and may play an important role in ligand−protein binding.[3] In contrast to the large conformational changes that have been shown to occur in proteins upon ligand binding, these small and subtle changes have not been fully appreciated and are typically ignored in current drug design practice.

It has generally been assumed that binding of ligands to proteins would decrease atomic mobility of proteins, especially at the ligand-binding sites (local protein flexibility). However, an increase in protein backbone conformational flexibility was observed upon binding of a small hydrophobic ligand to mouse major urinary protein,[4] and this raises the question of the generality of such effects. To the best of our knowledge, no systematic analysis has been carried out to study the effect of ligand binding on protein flexibility at ligand-binding sites. In this paper, we report our systematic analysis of the effect of ligand binding on protein flexibility, with a focus on ligand-binding sites. This was done with a diverse set of high-resolution crystal structures of proteins, both free and complexed with ligands.

To analyze the effect of ligand binding on protein flexibility of ligand-binding sites, we compared the atomic *B*-factors of binding-site protein atoms before and after ligand binding. The isotropic atomic *B*-factors reported in protein structures obtained from X-ray crystallographic analysis are based on a harmonic model[5] that reflects the positional spread of each atom by the relationship $B = 8\pi^2\overline{U^2}$, where $\overline{U^2}$ is the mean square of the displacement of an atom. Cartesian coordinates for each atom define the position where the atom can be found with maximal probability, and the atomic *B*-factor describes the extent of the vibration of the atom around this equilibrium position. The atomic *B*-factor includes information of both atomic mobility and intrinsic disorder of the structure and has been exploited in prediction of protein flexibility[6,7] and correlation of side chain mobility with conformation.[8] In our current analysis we have used the atomic *B*-factors as an indicator of the atomic mobility in crystal structures and, collectively, as an indicator of protein flexibility.

Our recent compilation[9] of 800 high-resolution protein−ligand complexes from the Protein Data Bank (PDB)[10] has provided us with a list of candidate proteins for this work. Using protein names from this compilation,[9] we searched the PDB to find the corresponding ligand-free protein structures to examine changes in atomic *B*-factors upon ligand binding.

The reported values of atomic *B*-factors in crystal structures in the PDB not only depend on the actual atomic mobility but also are influenced by the refinement strategies used.[11,12] Hence, normalized *B*-factors[8] had been used in the past when *B*-factors in different proteins were compared. However, a drawback using the normalized *B*-factors in our analysis is that different proteins may have different inherent plasticity, which is not reflected in the normalized *B*-factors. To overcome these limitations, we have adopted the following very stringent criteria in our selection of a high-quality data set for our study. (1) Both the ligand-free and the corresponding ligand-bound protein structure(s) must be determined by the same research group with the same refinement strategy used for ligand-free and ligand-bound structures. (2) The resolution of structures should be better than 2.5 Å except in the case of two ligand-free protein structures (2.7 and 2.75 Å) and two complex structures (2.6 and 2.7 Å). (3) The *R* values for all but two of the structures are less than 0.245 Å. With these selection criteria, we have identified a total of 63 protein−ligand complex structures and 37 corresponding ligand-free protein structures. The 37 proteins are from 24 known protein superfamilies based on the CATH classification[13] and 5 are unclassified proteins (see Supporting Information). These 63 complex structures contain 61 different ligands, including small organic molecules, sugars, and peptides with molecular weights ranging from 65 to 1200. Comparisons of the atomic *B*-factors between ligand-free and ligand-bound protein structures were then performed. The PDB codes, resolution, and *R* value of ligand-free and ligand-bound protein structures used in this study are provided in the Supporting Information. We defined the ligand-binding site in a protein as the region within 8 Å of any ligand atom in the protein−ligand complex structure and focused our analysis on these binding-site protein atoms. The results are summarized in Figure 1.

Upon ligand binding, 71% of the binding-site protein atoms become less mobile ($\Delta B < 0$) but 29% of binding-site protein atoms become more mobile ($\Delta B > 0$). This is reflected in the asymmetric distribution around zero

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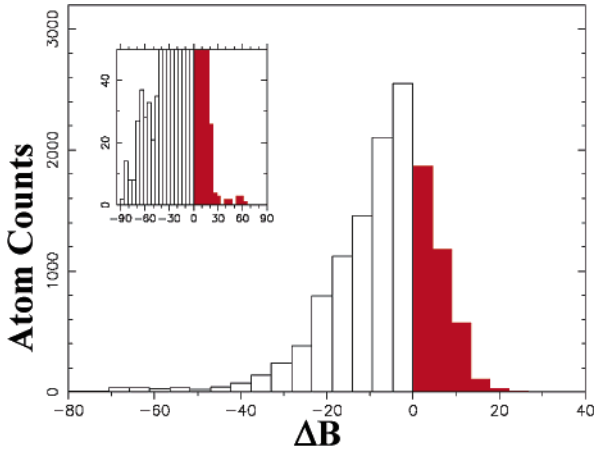

Figure 1. Histogram distribution of the changes of atomic *B*-factors upon ligand binding for protein atoms within 8 Å of any ligand atom.

in Figure 1, indicating that while the majority of atoms around the binding site become less mobile, a significant percentage of binding-site protein atoms actually become more mobile. At the half-height of the distribution for $\Delta B < 0$, the value of ΔB is -12. This corresponds to a reduction of 0.39 Å in atomic mobility, which is defined as $[B/(8\pi^2)]^{1/2}$ assuming isotropic motion. At the half-height of the distribution for $\Delta B > 0$, the value of ΔB is $+8$, which corresponds to an increase of 0.32 Å in atomic mobility. The tail of the distribution with $\Delta B < 0$ tapers off more slowly than those with $\Delta B > 0$ and reaches -90, in comparison to $+60$ (see the inset figure). It is noteworthy that a number of atoms show an exceptionally large increase or decrease in their *B*-factors upon ligand binding (see inset in Figure 1). A similar pattern of distribution was also observed when using the normalized *B*-factor for calculation of ΔB (see Figure S1 in Supporting Information).

In terms of magnitude in the *B*-factor changes for protein atoms in the ligand-binding sites, 14 complexes (1EHH, 1J3R, 1KLL, 1LAM, 1LRH, 1YET, 1D7H, 1F73, 1I05, 1LIC, 1OGE, 1D7J, 1KUK, and 1LIE) have an average absolute ΔB value of 4.16, indicating that the effect of ligand binding on protein flexibility is small for these proteins. However, the remaining 49 protein complexes show greater changes in their *B*-factors.

Atomic *B*-factors depend on the temperature at which the crystal structure is determined. To rule out the temperature effect in our analysis, we identified the subset from these 63 ligand−protein complex structures, which were determined at a temperature within ±3 K of that for the corresponding ligand-free protein structures; 47 structures were thus identified. The histogram distribution of the changes of *B*-factors upon ligand binding using the data of these 47 ligand−protein complex structures and their corresponding 31 ligand-free structures showed a pattern (see Supporting Information, Figure S3) very similar to that in Figure 1. This indicates that the changes in atomic *B*-factors upon ligand binding for protein atoms of the ligand-binding sites do not result from the different temperatures used for structure determination. Another 16 ligand−protein complex structures and their corresponding ligand-free structures, determined at somewhat different temperatures ($\Delta T > 3$ K or the information is not available), also show a similar pattern in their histogram distribution of the changes of *B*-factors upon ligand binding to that observed for the 47 ligand−protein complex structures and their corresponding ligand-free structures.

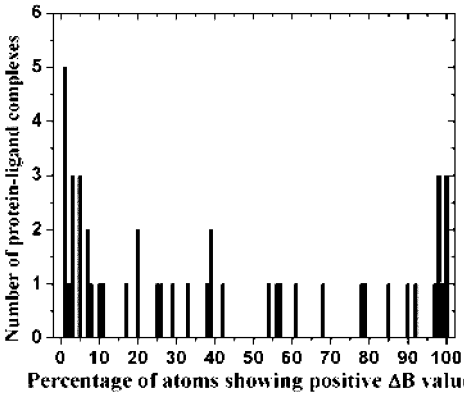

Figure 2. Number of protein−ligand complexes with various percentages of protein atoms at the ligand binding sites exhibiting an increase in their *B*-factors after ligand binding.

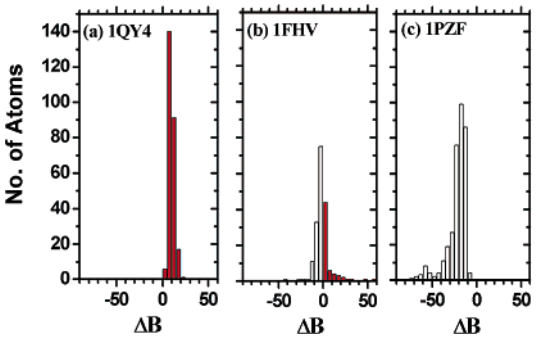

Figure 3. Three complexes with protein atoms at the binding sites showing (a) all positive, (b) mixed positive and negative, and (c) all negative changes of *B*-factors upon ligand binding. The *x*-axis is ΔB, and the *y*-axis is the number of atoms. The PDB code is shown in the top-left corner of each plot.

To investigate whether the *B*-factor increases upon ligand binding are only associated with a relatively few complexes, we calculated the percentage of binding-site atoms with $\Delta B > 0$ in each complex. As can be seen from Figure 2, a majority of the proteins have certain percentages of atoms exhibiting an increased *B*-factor upon ligand binding; 47 of the 63 complexes show positive values of ΔB for at least some binding-site protein atoms. It was found that three complex structures (1KKR, 1QTE, and 1QY4), all determined at the same temperature as the corresponding free protein structures, exhibit an increase in the *B*-factor upon ligand binding for all protein atoms in the binding site (Figure 3). These three cases all involve proteins with metal ions in the protein structures and participating in ligand binding. Hence, the global increase in protein flexibility of the ligand-binding sites in these three cases may have resulted from disruption of the strong interaction of the metal ion with other protein atoms upon ligand binding. We also found that 16 complexes (1ALW, 1FB8, 1FKJ, 1I0S, 1I3A, 1EN2, 1F74, 1JYR, 1OGD, 1OGF, 1PZF, 1PZG, 1PZH, 4ENL, 5ENL, and 6ENL) show a global decrease in *B*-factors upon ligand binding for all protein atoms in the ligand-binding sites. Two proteins exhibiting a completely opposite change in ΔB are displayed in Figure 4.

Proteins belonging to the same superfamily such as the lipocalins (1G74, 1HBP, 1I05, 1I06, 1KQW, 1LIC,

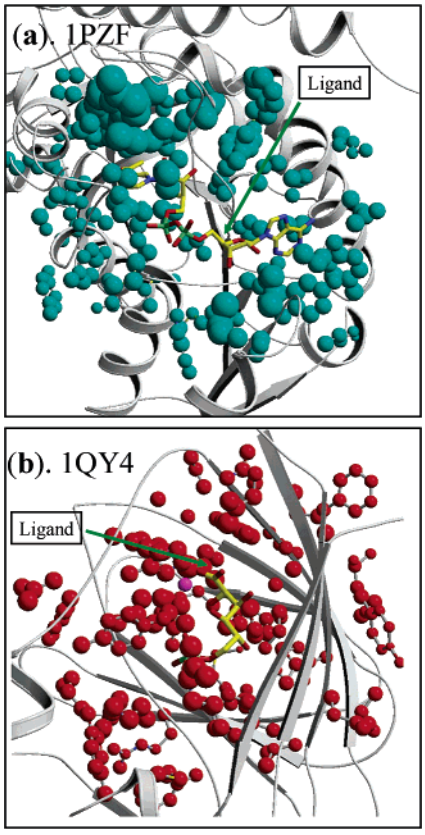

Figure 4. Two proteins exhibit different overall changes of *B*-factors upon ligand binding. While an overall reduction of *B*-factors (<0) was found in (a) for 1PZF, the opposite (>0) was found in (b) for 1QY4. The magnitudes of ΔB for protein side chain atoms within 8 Å from the ligand were represented by the ball sizes, with the cyan and red colors indicating $\Delta B > 0$ and $\Delta B < 0$, respectively. Backbone atoms are omitted for clarity. A nickel ion in 1QY4 is shown by a purple ball.

1LID, 1LIE, 1LIF, and 1OPB) show different patterns in the *B*-factor changes when bound to different ligands. Even for the same protein, different ligands can lead to different patterns of the *B*-factor changes, ranging from mixed positives and negatives to all negatives (e.g., 1F73, 1F74, and 1F7B).

We also found that three protein structures (1JYQ, 1JYR, and 1KLL) undergo significant backbone conformational changes upon ligand binding. Two of these three structures (1JYQ and 1JYR) become more compact compared to their corresponding ligand-free structures and show a wide range of decreases in *B*-factors upon ligand binding.

We further analyzed individual amino acid residues around the ligand-binding sites that showed large changes in their *B*-factors ($\Delta B > 12$ or $\Delta B < -24$) upon ligand binding. It was found that very few backbone protein atoms show a large increase in their *B*-factors. On average, the backbone atoms of Glu, Arg, and Ser residues experience the largest reduction in their *B*-factors upon ligand binding. The reasons are not entirely clear, but the large reduction in *B*-factors for these backbone atoms may be attributable to the ability to form hydrogen bonds with ligands through the Glu, Arg, and Ser side chains. In contrast to backbone atoms, a significant number of side chain atoms in Arg, Leu, Tyr, Lys, Glu, and His residues show a large increase in their *B*-factors upon ligand binding. Only a limited number of side chain atoms in Asp, Gln, Ser, Thr, Met, and Phe residues, however, show a large increase in their *B*-factors. Very few side chain atoms in Pro, Cys, Ile, and Ala show either a large increase or decrease in their *B*-factors. Interestingly, for Glu and Arg residues, there appears to be an opposite change for the backbone (large decreases in their *B*-factors) and side chain atoms (large increases in their *B*-factors) upon ligand binding.

In summary, upon ligand binding, 47 of the 63 complexes studied (75%) show an increase in their *B*-factors for at least some protein atoms around the binding sites. Of the protein atoms in the binding site, 71% become less mobile and 29% more mobile upon ligand binding. Binding leads to a more rigid structure in 16 proteins but to a more flexible structure in 3 of the proteins studied. Few backbone atoms show large increases in their *B*-factors, but a significant number of side chain atoms, especially in Arg, Leu, Tyr, Lys, Glu, and His, show large increases.

This systematic analysis demonstrates that binding of small-molecule ligands to proteins does not always lead to a reduction of local protein flexibility and may in fact result in a significant increase in the mobility of some binding-site protein atoms, especially for some side chain atoms. In a few cases, ligand binding to a protein actually leads to a global increase in protein local flexibility. The observed increases and decreases in local protein flexibility of the ligand-binding sites upon ligand binding may be significant for protein function and may have important implications for structure-based design of small-molecule ligands.

Supporting Information Available: Reference and details for each structure used in this paper and analysis of the *B*-factor changes for each protein−ligand complex structure in comparison to its ligand-free structure. This material is available free of charge via the Internet at http://pubs.acs.org.

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