Supporting information for:
Attach-pull-release calculations of ligand binding and conformational changes on the first BRD4 bromodomain

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1 Supplementary Methods

1.1 Restraint setup and calculations

The chosen protein anchors are the C-α atoms from residues Ile110, Thr131 and Phe157, and the anchor atoms of each ligand are chosen based on its structure and binding mode (Fig. 3). The reference values of the various harmonic potentials are obtained directly from the initial setup of Fig. 1a, with the positioning of the three non-interacting anchor particles depending on the ligand. They are arranged so that they are always on the plane defined by the N1, L1 and P1 anchors (YZ plane); the D1 and D2 distances are always parallel to the z-axis; and the reference value of the A1 and A3 angles is always 90 degrees. Also, the initial value of D1 in the bound state is 5.0 Å, and the final distance after the pulling is 21.0 Å.

All distance restraints in this study used force constants of \( k_d = 5\text{kcal/mol.Å}^2 \). Angle restraints A1 to A4, torsions T1 to T6, and the ligand dihedral restraints, when applied, used force constants of \( k_a = 100\text{kcal/mol.rad}^2 \). The force constant applied to the Asp88N-Asp88CA-Asp88C-Ala89N dihedral is \( k_t = 20\text{kcal/mol.rad}^2 \), and that for the 20 dihedrals attached in the conformational calculations of the apo protein the force constant is \( k_c = 50\text{kcal/mol.rad}^2 \). It is worth noting that, according to the AMBER definitions, the force applied to a given coordinate \( x \) is given by \( F = -2k(x - x_0) \), with \( x_0 \) being its reference value. During the attachment stages using MBAR, we simulate systems with intermediate values \( k' \) of the spring constants outlined above, such that \( k' = \lambda k \), with \( \lambda \) varying in \( K = 15 \) steps, or windows, from zero to one. The difference between two adjacent \( \lambda \) values, \( \lambda_i \) and \( \lambda_{i+1} \), was set to grow exponentially with the window number \( i \) until \( \lambda = 1 \). This generates a higher density of windows for values of \( \lambda \) closer to zero, where fluctuations tend to be larger. The releasing stages use the same 15 windows and \( \lambda_i \) values, but starting at \( \lambda = 1 \) and running to \( \lambda = 0 \).

Table S1 summarizes the restraints that are attached, released, or held constant during
each step of the binding free energy calculations, both for the protein and the ligand. We also indicate which elements are present in the simulation box, the number of restraints being modified during the step ($R$ in Eq. 2), and the corresponding panel from Fig. 2.
Table S1: Details of restraints applied, removed, changed, or held constant during the various stages of the APR calculations, with the corresponding panels from Fig. 2 (from the main paper) shown in the sixth column, and the quantities computed in the final column (Symbol).

| Phase      | Molecules in the box | Constant Restraints | Changing Restraints | R value (Eq. 2) | Notes                     | Symbol            |
|------------|----------------------|---------------------|---------------------|-----------------|---------------------------|-------------------|
| **Attach** | Protein and bound ligand | None                | P1-P2-P3 distances and Asp88 ψ dihedral | 4               | Top panel, step 1         | ΔG_{attach,p}    |
|            |                      |                     |                     |                 |                           |                   |
| **Ligand attach** |                      | All protein restraints* | All ligand restraints† | 9 (lig. 1, 2) | Top panel, step 2         | ΔG_{attach,l}    |
| **Pull**   | Protein and bound ligand | All restraints*† except D1 | D1 restraint reference value | 1               | Top panel, step 3         | ΔG_{pull}         |
| **Release** | Ligand only          | None                | All ligand restraints† | 3 (lig. 1, 2)† | Middle panel (inset)      | ΔG_{release,l}   |
|            | Protein only         |                      |                     |                 |                           |                   |
| **Protein release to apo open state** |                  | P1-P2-P3 distances and Asp88 ψ dihedral | 20 additional dihedral restraints (Fig. 1b) | 20              | Middle panel, the three contributions add up to ΔG_{release,p} | ΔG_{conf,attach} |
|            |                      | P1-P2-P3 distances and the 20 extra dihedrals | Asp88 ψ dihedral restraint reference value | 1               |                           | ΔG_{conf,pull}   |
|            |                      | None                | P1-P2-P3 distances, and all the 21 dihedrals | 24              |                           | ΔG_{conf,release}|
| **Protein release to apo closed state** |                  | Asp88 ψ dihedral wall only | P1-P2-P3 distances and Asp88 ψ dihedral | 4               | Bottom panel               | ΔG_{release,p,cl}|

* P1-P2-P3 distances, Asp88 ψ dihedral, D2, A3, A4, T4, T5, T6 (Fig. 1a).
† L1-L2-L3 distances, ligand dihedral when present, D1, A1, A2, T1, T2, T3 (Fig. 1a).
‡ The six rigid-body restraints are removed analytically using Eq. 3 from the main paper.
1.2 Multistate Bennett Acceptance Ratio

We use the Multistate Bennett Acceptance Ratio (MBAR)\textsuperscript{S1} method to obtain all the contributions to the standard binding free energy $\Delta G_{\text{bind}}^0$, except for $(\Delta G_{\text{release, l-std}})$, which is calculated semi-analytically as described in the Methods section. In the MBAR approach, we separate the initial and final states of a given calculation, for example the unrestrained and the fully attached ligand in the binding site, into intermediate windows, and combine them to obtain the best estimate for the free energy difference between these two states. This is done by calculating the total restraint potential energy of a given simulation frame using the restraint potentials of each window, and combining them through the expression:

$$G_i = -\beta^{-1} \sum_{j=1}^{K} \sum_{n=1}^{N_j} \frac{\exp(-\beta U_i(x_{nj}))}{\sum_{k=1}^{K} N_k \exp(\beta G_k - \beta U_k(x_{nj}))}$$

Here, $G_i$ is the free energy of window $i$, and $G_k$ is the free energy of window $k$, with $\beta = 1/k_B T$. $K$ is the total number of simulation windows, which can be defined in terms of a varying reaction coordinate, as in the pulling stage; or varying spring constants applied to a set of distances, angles and dihedrals, as in the attaching and releasing phases. The index $n$ identifies a given simulation frame from window $j$, and $N_j$ is the total number of samples from this window. The term $U_i(x_{nj})$ is the potential energy from all harmonic restraints from window $i$ acting on the set of restrained coordinates $x_{nj}$, obtained from the $n$th sample from window $j$. We define $U_i(x_{nj})$ as:

$$U_i(x_{nj}) = \sum_{r=1}^{R} k_{ir} (x_{njr} - x_{0,ir})^2,$$

where $k_{ir}$ and $x_{0,ir}$ are, respectively, the spring constant and reference value of the harmonic potential of the $r$th restraint from window $i$, with $R$ being the total number of restrained degrees of freedom included in the calculation (see Table S1). $U_k(x_{nj})$ has the same form as Eq. 2, with $N_k$ being the total number of samples of window $k$. After post-processing
all the simulations to obtain the potentials $U_i$ and $U_k$ at each frame, we use the program Pymbar$^5$ to solve Eq. 1 self-consistently yielding a set of free energies $G_i$. This set is defined only to within an additive constant, which can be ignored since we are only looking for free energy differences. Their associated uncertainties are also provided by the Pymbar program, from a covariance matrix that includes only the uncorrelated samples from each window.$^5$ These uncorrelated samples are obtained by subsampling the correlated data from the simulations based on their statistical inefficency, which in turn is determined using the integrated autocorrelation time from a given time series. Since the uncertainty provided by MBAR is the standard deviation of the mean, the uncertainties of the terms in Eq. 1 may be added in quadrature (equivalent to adding the variances) to obtain the final uncertainty of a given process.

1.3 BRD4 conformation in the restrained states

The restraints on overall protein translation and rotation are designed not to influence the conformational distribution of the protein, so that the work of imposing or removing them does not need to be computed. We checked this empirically by comparing simulations with only the protein conformational restraints, which are the three P1-P2-P3 distance restraints and the Asp 88 $\psi$ dihedral in the closed state, present against matched simulations that additionally include the translational and rotational restraints on the protein. First, Fig. S1 compare the final structures (A,B) and backbone RMSD values computed relative to the time-averaged structures (C,D) from 200 ns simulations, each started from a 100 ns equilibrated system, with only the conformational restraints attached, and with the translational and rotational restraints also present. (For the backbone RMSD calculations we exclude the highly flexible N-terminal stretch from BRD4, since the protein anchor atoms are all located in the more rigid inner helices, and the fluctuations of that loop could interfere in the results.) Panels A and C show results for the apo protein, and panels B and D show results for the protein with ligand 3 bound. There are only minimal differences between
structures in the presence and absence of translational and rotational restraints (A, B), and the backbone RMSD values also agree well. Second, using the same set of simulations, we compared the distributions of the backbone torsion angles of the three residues that have their C-α atoms as anchors: Ile110, Thr131 and Phe157 (Fig. S2). Again, no significant influence of the translational and rotational restraints is seen.
Figure S1: (A): Comparison of BRD4 bromodomain apo structures after 300 ns with only conformational restraints applied (red) and with all the conformational, translational and rotational restraints applied to the protein (blue). The anchor atoms are indicated in yellow, and the transparent section is the N-terminal loop, which is not included in the RMSD calculations. (B): Same as (A) for BRD4 bound to ligand 3, also showing the substrate molecule. (C): Histograms for the RMSD (Å) of the apo BRD4 during 200 ns of simulation, in the two restraint regimes. The black bars indicate the state with only conformational restraints applied, and the red lines the fully restrained state. (D): Same as (C), but for the bound state of BRD4 with ligand 3.
Figure S2: (A,C,E): Each graph shows probability distributions of $\phi$ and $\psi$ torsion angles for one of the three residues that contain the anchor atoms in the apo protein. Black and green are the $\phi$ dihedrals for the conformationally restrained and the fully restrained state, respectively, and red and blue the same for the $\psi$ dihedral. All angles are in degrees. (B,D,F): Same for the protein bound to ligand 3.
Figure S3: Same graphs as Fig. 7a, analyzing the time evolution of the four key torsion angles (hinges) involved in the conformational transition of the apo BRD4. Simulations are for the two apo crystal structures (4LYI and 2OSS) and the three water models. The transition happens spontaneously in all cases.
Figure S4: Histogram showing the values of the Asp88 $\psi$ dihedral in the apo state of the BRD4 bromodomain, when it is restrained to the closed conformation. We can see that the presence of the wall does not interfere with the harmonic restraints, and therefore it can be safely used to release the apo BRD4 to the metastable closed state.
Figure S5: (A) Comparison of the 4LYS crystal structure of the BRD4 bromodomain bound to the XD1 ligand (red), with the second binding pose obtained by docking the same ligand to the 4LYI apo structure (green). (B) The other two poses obtained for XD1 from the docking to the apo structure: pose ranked number 1 shown in yellow, and pose ranked number 3 in purple. (C) The two poses of the RVX-208 ligand from the 4J3I crystal structure, 4J3I-A in red and 4J3I-B in green. (D) Comparison of the 4J3I-A pose of RVX-208 (red) with the one obtained based on the RVX-OH complex with BRD4 (yellow), from the 4MR3 crystal structure.
## Supplementary Results

Table S2: Computed standard binding free energies of the 7 ligands to the BRD4 bromodomain, both for the closed ($\Delta G_{\text{bind,closed}}$) and the open ($\Delta G_{\text{bind}}$) final states of the apo protein, along with the contributions from each step in the APR procedure, from simulations using TIP3P and GAFF. Experimental data are provided in column 2. Results are in kcal/mol. The values in parenthesis are the associated uncertainties.

| Lig. | $\Delta G_{\text{exp}}$ | $\Delta G_{\text{bd,cl}}$ | $\Delta G_{\text{bind}}$ | $\Delta G_{\text{at,p}}$ | $\Delta G_{\text{at,l}}$ | $\Delta G_{\text{pull}}$ | $\Delta G_{\text{rl,l}}$ | $\Delta G_{\text{rl,p}}$ | $\Delta G_{\text{rl,p,cl}}$ |
|------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| 1    | $-5.95^a$ (ND)          | $-7.13$ (0.11)          | $-4.59$ (0.16)          | $1.83$ (0.03)           | $5.06$ (0.06)           | $11.84$ (0.09)          | $-10.17$ (0.01)         | $-3.97$ (0.12)          | $-1.43$ (0.02)          |
| 2    | $-6.36^b$ (0.10)        | $-5.59$ (0.13)          | $-3.05$ (0.17)          | $1.67$ (0.03)           | $6.09$ (0.07)           | $9.37$ (0.11)           | $-10.11$ (0.01)         | $-3.97$ (0.12)          | $-1.43$ (0.02)          |
| 3    | $-7.01^b$ (ND)          | $-7.35$ (0.15)          | $-4.81$ (0.19)          | $1.50$ (0.02)           | $5.88$ (0.06)           | $13.18$ (0.13)          | $-11.78$ (0.03)         | $-3.97$ (0.12)          | $-1.43$ (0.02)          |
| 4    | $-8.16^a$ (0.03)        | $-10.07$ (0.20)         | $-7.53$ (0.23)          | $1.89$ (0.03)           | $7.40$ (0.08)           | $14.61$ (0.18)          | $-12.40$ (0.04)         | $-3.97$ (0.12)          | $-1.43$ (0.02)          |
| 5    | $-8.99^a$ (0.07)        | $-8.89$ (0.17)          | $-6.35$ (0.21)          | $1.84$ (0.03)           | $5.21$ (0.07)           | $15.38$ (0.13)          | $-12.11$ (0.06)         | $-3.97$ (0.12)          | $-1.43$ (0.02)          |
| 6    | $-9.45^b$ (0.13)        | $-8.16$ (0.20)          | $-5.62$ (0.23)          | $1.75$ (0.03)           | $5.75$ (0.08)           | $14.08$ (0.14)          | $-11.99$ (0.11)         | $-3.97$ (0.12)          | $-1.43$ (0.02)          |
| 7    | $-10.41^b$ (0.23)       | $-9.11$ (0.17)          | $-6.57$ (0.20)          | $1.84$ (0.03)           | $8.51$ (0.11)           | $12.28$ (0.12)          | $-12.09$ (0.03)         | $-3.97$ (0.12)          | $-1.43$ (0.02)          |

$^a$ Obtained by isothermal titration calorimetry. $^b$ Obtained using Alphascreen. ND - Uncertainty value not determined by experiments.
Table S3: Same as Table S2, but for TIP3P and GAFF2.

| Lig. | $\Delta G_{\text{exp}}^0$ | $\Delta G_{\text{bind}}^{0,cl}$ | $\Delta G_{\text{bind}}^0$ | $\Delta G_{at,p}$ | $\Delta G_{at,l}$ | $\Delta G_{\text{pull}}$ | $\Delta G_{rl,l}$ | $\Delta G_{rl,p}$ | $\Delta G_{rl,p,cl}$ |
|------|------------------------|-------------------------------|----------------------------|-----------------|-----------------|------------------|----------------|----------------|------------------|
| 1    | -5.95 (ND)             | -7.21 (0.13)                 | -4.67 (0.17)              | 1.87 (0.03)     | 4.89 (0.06)     | 12.04 (0.11)    | -10.16 (0.01)  | -3.97 (0.12)   | -1.43 (0.02)    |
| 2    | -6.36 (0.10)           | -5.19 (0.13)                 | -2.65 (0.18)              | 1.69 (0.03)     | 6.14 (0.07)     | 8.92 (0.11)     | -10.13 (0.01)  | -3.97 (0.12)   | -1.43 (0.02)    |
| 3    | -7.01 (ND)             | -6.98 (0.16)                 | -4.44 (0.20)              | 1.35 (0.02)     | 5.80 (0.07)     | 13.46 (0.14)    | -12.20 (0.04)  | -3.97 (0.12)   | -1.43 (0.02)    |
| 4    | -8.16 (0.03)           | -10.61 (0.21)                | -8.07 (0.24)              | 1.81 (0.03)     | 7.13 (0.09)     | 15.10 (0.18)    | -12.00 (0.04)  | -3.97 (0.12)   | -1.43 (0.02)    |
| 5    | -8.99 (0.07)           | -7.71 (0.25)                 | -5.17 (0.28)              | 1.90 (0.03)     | 5.21 (0.06)     | 13.87 (0.23)    | -11.84 (0.06)  | -3.97 (0.12)   | -1.43 (0.02)    |
| 6    | -9.45 (0.13)           | -7.21 (0.14)                 | -4.67 (0.18)              | 1.80 (0.03)     | 5.66 (0.07)     | 12.89 (0.10)    | -11.71 (0.04)  | -3.97 (0.12)   | -1.43 (0.02)    |
| 7    | -10.41 (0.23)          | -9.43 (0.16)                 | -6.89 (0.20)              | 1.79 (0.03)     | 6.19 (0.07)     | 14.26 (0.14)    | -11.38 (0.02)  | -3.97 (0.12)   | -1.43 (0.02)    |
Table S4: Same as Table S2, but for TIP4PEw and GAFF.

| Lig. | $\Delta G^0_{\text{exp}}$ | $\Delta G^0_{\text{bind},\text{cl}}$ | $\Delta G^0_{\text{bind}}$ | $\Delta G_{\text{at},\text{p}}$ | $\Delta G_{\text{at},l}$ | $\Delta G_{\text{pull}}$ | $\Delta G_{\text{rl},l}$ | $\Delta G_{\text{rl},p}$ | $\Delta G_{\text{rl},p,cl}$ |
|------|--------------------------|----------------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| 1    | -5.95 (ND)               | -7.95 (0.19)                    | -4.23 (0.26)            | 2.01 (0.03)               | 5.18 (0.07)               | 12.70 (0.17)             | -10.19 (0.01)            | -5.47 (0.17)             | -1.75 (0.03)             |
| 2    | -6.36 (0.10)             | -5.65 (0.17)                    | -1.93 (0.24)            | 1.81 (0.03)               | 5.72 (0.08)               | 9.98 (0.14)              | -10.11 (0.01)            | -5.47 (0.17)             | -1.75 (0.03)             |
| 3    | -7.01 (ND)               | -6.36 (0.16)                    | -2.64 (0.24)            | 1.52 (0.03)               | 5.72 (0.07)               | 12.65 (0.13)             | -11.78 (0.03)            | -5.47 (0.17)             | -1.75 (0.03)             |
| 4    | -8.16 (0.03)             | -11.77 (0.19)                   | -8.05 (0.26)            | 2.03 (0.04)               | 7.57 (0.09)               | 16.27 (0.16)             | -12.35 (0.04)            | -5.47 (0.17)             | -1.75 (0.03)             |
| 5    | -8.99 (0.07)             | -9.76 (0.30)                    | -6.04 (0.35)            | 1.88 (0.03)               | 5.14 (0.06)               | 16.45 (0.29)             | -11.96 (0.06)            | -5.47 (0.17)             | -1.75 (0.03)             |
| 6    | -9.45 (0.13)             | -9.27 (0.26)                    | -5.55 (0.31)            | 1.84 (0.03)               | 6.20 (0.08)               | 14.95 (0.21)             | -11.97 (0.12)            | -5.47 (0.17)             | -1.75 (0.03)             |
| 7    | -10.41 (0.23)            | -11.44 (0.26)                   | -7.72 (0.32)            | 1.83 (0.03)               | 10.19 (0.20)              | 13.23 (0.16)             | -12.06 (0.03)            | -5.47 (0.17)             | -1.75 (0.03)             |
Table S5: Same as Table S2, but for TIP3PEw and GAFF2.

| Lig. | $\Delta G_{exp}^0$ | $\Delta G_{bind}^{0,cl}$ | $\Delta G_{bind}^0$ | $\Delta G_{at,p}$ | $\Delta G_{at,l}$ | $\Delta G_{pull}$ | $\Delta G_{rl,l}$ | $\Delta G_{rl,p}$ | $\Delta G_{rl,p,cl}$ |
|------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| 1    | -5.95 (ND)       | -7.87 (0.17)     | -4.15 (0.24)     | 2.10 (0.04)      | 4.94 (0.06)      | 12.74 (0.15)     | -10.16 (0.01)    | -5.47 (0.17)     | -1.75 (0.03)     |
| 2    | -6.36 (0.10)     | -5.44 (0.16)     | -1.72 (0.24)     | 1.75 (0.03)      | 6.13 (0.08)      | 9.44 (0.14)      | -10.13 (0.01)    | -5.47 (0.17)     | -1.75 (0.03)     |
| 3    | -7.01 (ND)       | -6.50 (0.19)     | -2.78 (0.26)     | 1.44 (0.02)      | 5.83 (0.07)      | 13.23 (0.17)     | -12.25 (0.04)    | -5.47 (0.17)     | -1.75 (0.03)     |
| 4    | -8.16 (0.03)     | -11.54 (0.25)    | -7.82 (0.31)     | 1.92 (0.03)      | 7.10 (0.08)      | 16.32 (0.23)     | -12.05 (0.04)    | -5.47 (0.17)     | -1.75 (0.03)     |
| 5    | -8.99 (0.07)     | -10.10 (0.19)    | -6.38 (0.26)     | 1.88 (0.03)      | 5.14 (0.06)      | 16.83 (0.17)     | -12.00 (0.07)    | -5.47 (0.17)     | -1.75 (0.03)     |
| 6    | -9.45 (0.13)     | -9.61 (0.17)     | -5.89 (0.25)     | 1.90 (0.03)      | 5.98 (0.08)      | 15.28 (0.12)     | -11.80 (0.09)    | -5.47 (0.17)     | -1.75 (0.03)     |
| 7    | -10.41 (0.23)    | -11.68 (0.18)    | -7.96 (0.26)     | 1.86 (0.03)      | 6.49 (0.07)      | 16.51 (0.16)     | -11.43 (0.03)    | -5.47 (0.17)     | -1.75 (0.03)     |
Table S6: Same as Table S2, but for SPC/E and GAFF.

| Lig. | $\Delta G^0_{\text{exp}}$ | $\Delta G^0_{\text{bind}}$ | $\Delta G^0_{\text{bind},cl}$ | $\Delta G_{\text{at},p}$ | $\Delta G_{\text{at},l}$ | $\Delta G_{\text{pull}}$ | $\Delta G_{\text{rl},l}$ | $\Delta G_{\text{rl},p}$ | $\Delta G_{\text{rl},p,cl}$ |
|------|----------------|-----------------|-----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| 1    | -5.95 (ND) | -7.60 (0.17) | -5.93 (0.27) | 1.69 (0.03) | 5.01 (0.06) | 12.82 (0.15) | -10.19 (0.01) | -3.40 (0.21) | -1.73 (0.03) |
| 2    | -6.36 (0.10) | -5.49 (0.15) | -3.82 (0.26) | 1.70 (0.03) | 6.06 (0.07) | 9.57 (0.13) | -10.11 (0.01) | -3.40 (0.21) | -1.73 (0.03) |
| 3    | -7.01 (ND) | -6.84 (0.19) | -5.17 (0.28) | 1.44 (0.02) | 5.64 (0.07) | 13.25 (0.17) | -11.76 (0.03) | -3.40 (0.21) | -1.73 (0.03) |
| 4    | -8.16 (0.03) | -10.89 (0.17) | -9.22 (0.27) | 1.96 (0.03) | 7.09 (0.08) | 16.00 (0.13) | -12.43 (0.05) | -3.40 (0.21) | -1.73 (0.03) |
| 5    | -8.99 (0.07) | -9.79 (0.18) | -8.12 (0.28) | 1.71 (0.03) | 5.05 (0.06) | 16.93 (0.15) | -12.17 (0.06) | -3.40 (0.21) | -1.73 (0.03) |
| 6    | -9.45 (0.13) | -9.98 (0.21) | -8.31 (0.30) | 1.77 (0.03) | 5.91 (0.07) | 16.53 (0.17) | -12.50 (0.10) | -3.40 (0.21) | -1.73 (0.03) |
| 7    | -10.41 (0.23) | -11.03 (0.20) | -9.36 (0.29) | 1.82 (0.03) | 8.47 (0.10) | 14.49 (0.16) | -12.02 (0.03) | -3.40 (0.21) | -1.73 (0.03) |
Table S7: Same as Table S2, but for SPC/E and GAFF2.

| Lig. | $\Delta G^0_{\text{exp}}$ | $\Delta G^0_{\text{bind}}$ | $\Delta G^0_{\text{bind}}$ | $\Delta G_{\text{at,p}}$ | $\Delta G_{\text{at,l}}$ | $\Delta G_{\text{pull}}$ | $\Delta G_{\text{rl,l}}$ | $\Delta G_{\text{rl,p}}$ | $\Delta G_{\text{rl,p,cl}}$ |
|------|-----------------|----------------|----------------|-----------------|----------------|----------------|----------------|----------------|----------------|
| 1    | -5.95 (ND)      | -6.87 (0.19)  | -5.20 (0.28)  | 1.84 (0.03)     | 4.63 (0.06)   | 12.30 (0.18)  | -10.17 (0.01) | -3.40 (0.21) | -1.73 (0.03)  |
| 2    | -6.36 (0.10)    | -5.31 (0.17)  | -3.64 (0.27)  | 1.59 (0.03)     | 6.12 (0.07)   | 9.46 (0.15)   | -10.13 (0.01) | -3.40 (0.21) | -1.73 (0.03)  |
| 3    | -7.01 (ND)      | -7.37 (0.25)  | -5.70 (0.33)  | 1.55 (0.03)     | 5.68 (0.07)   | 14.08 (0.24)  | -12.21 (0.04) | -3.40 (0.21) | -1.73 (0.03)  |
| 4    | -8.16 (0.03)    | -10.93 (0.20) | -9.26 (0.29)  | 2.03 (0.03)     | 6.99 (0.08)   | 15.61 (0.18)  | -11.97 (0.04) | -3.40 (0.21) | -1.73 (0.03)  |
| 5    | -8.99 (0.07)    | -10.00 (0.20) | -8.33 (0.29)  | 1.74 (0.03)     | 5.06 (0.07)   | 16.76 (0.18)  | -11.83 (0.06) | -3.40 (0.21) | -1.73 (0.03)  |
| 6    | -9.45 (0.13)    | -9.51 (0.24)  | -7.84 (0.32)  | 1.71 (0.03)     | 5.81 (0.08)   | 15.70 (0.21)  | -11.98 (0.09) | -3.40 (0.21) | -1.73 (0.03)  |
| 7    | -10.41 (0.23)   | -9.75 (0.19)  | -8.08 (0.28)  | 1.71 (0.03)     | 6.32 (0.07)   | 14.91 (0.16)  | -11.46 (0.02) | -3.40 (0.21) | -1.73 (0.03)  |
Table S8: Same as Table 1 in main text, but including uncertainties (SEM), in parentheses.

| Ligand | Exp. | TIP3P-GAFF | TIP3P-GAFF2 | TIP4P-GAFF | TIP4P-GAFF2 | SPC/E-GAFF | SPC/E-GAFF2 |
|--------|------|------------|------------|------------|------------|------------|------------|
| 1      | -5.95<sup>a</sup> (ND)<sup>S2</sup> | -4.59 (0.16) | -4.67 (0.17) | -4.23 (0.26) | -4.15 (0.24) | -5.93 (0.27) | -5.20 (0.28) |
| 2      | -6.36<sup>b</sup> (0.10)<sup>S3,S4</sup> | -3.05 (0.17) | -2.65 (0.18) | -1.93 (0.24) | -1.72 (0.24) | -3.82 (0.26) | -3.64 (0.27) |
| 3      | -7.01<sup>b</sup> (ND)<sup>S5</sup> | -4.81 (0.19) | -4.44 (0.20) | -2.64 (0.24) | -2.78 (0.26) | -5.17 (0.28) | -5.70 (0.33) |
| 4      | -8.16<sup>a</sup> (0.03)<sup>S6</sup> | -7.53 (0.23) | -8.07 (0.24) | -8.05 (0.26) | -7.82 (0.31) | -9.22 (0.27) | -9.26 (0.29) |
| 5      | -8.99<sup>a</sup> (0.07)<sup>S7</sup> | -6.35 (0.21) | -5.17 (0.28) | -6.04 (0.35) | -6.38 (0.26) | -8.12 (0.28) | -8.33 (0.29) |
| 6      | -9.45<sup>b</sup> (0.13)<sup>S8</sup> | -5.62 (0.23) | -4.67 (0.18) | -5.55 (0.31) | -5.89 (0.25) | -8.31 (0.30) | -7.84 (0.32) |
| 7      | -10.41<sup>b</sup> (0.23)<sup>S9</sup> | -6.57 (0.20) | -6.89 (0.20) | -7.72 (0.32) | -7.96 (0.26) | -9.36 (0.29) | -8.08 (0.28) |

Statistics

|        | MSE | RMSE | y-int | slope | r   | τ   |
|--------|-----|------|-------|-------|-----|-----|
|        | 0.00 (0.06) | 0.14 (0.07) | -0.02 (0.39) | 1.00 (0.05) | 1.00 (0.01) | 0.89 (0.11) |
|        | 2.54 (0.44) | 2.77 (0.39) | -0.49 (2.81) | 0.63 (0.34) | 0.69 (0.25) | 0.43 (0.27) |
|        | 2.82 (0.58) | 3.18 (0.46) | -0.67 (3.43) | 0.58 (0.42) | 0.55 (0.27) | 0.33 (0.27) |
|        | 2.88 (0.56) | 3.21 (0.45) | 3.42 (3.76) | 1.07 (0.45) | 0.73 (0.21) | 0.40 (0.24) |
|        | 2.80 (0.53) | 3.10 (0.46) | 4.17 (3.64) | 1.18 (0.43) | 0.78 (0.19) | 0.44 (0.24) |
|        | 0.91 (0.43) | 1.42 (0.29) | 1.70 (3.03) | 1.10 (0.43) | 0.83 (0.16) | 0.49 (0.23) |
|        | 1.18 (0.46) | 1.66 (0.30) | 0.91 (3.54) | 0.98 (0.43) | 0.77 (0.25) | 0.38 (0.29) |
Table S9: Same as Table 2 in main text, but including uncertainties (SEM), in parentheses.

| Ligand | Exp. | TIP3P-GAFF | TIP3P-GAFF2 | TIP4P-GAFF | TIP4P-GAFF2 | SPC/E-GAFF | SPC/E-GAFF2 |
|--------|------|------------|-------------|------------|-------------|------------|-------------|
| 1      | −5.95\textsuperscript{a} (ND)\textsuperscript{S2} | −7.13 (0.11) | −7.21 (0.13) | −7.95 (0.19) | −7.87 (0.17) | −7.60 (0.17) | −6.87 (0.19) |
| 2      | −6.36\textsuperscript{b} (0.10)\textsuperscript{S3,S4} | −5.59 (0.13) | −5.19 (0.13) | −5.65 (0.17) | −5.44 (0.16) | −5.49 (0.15) | −5.31 (0.17) |
| 3      | −7.01\textsuperscript{b} (ND)\textsuperscript{S5} | −7.35 (0.15) | −6.98 (0.16) | −6.36 (0.16) | −6.50 (0.19) | −6.84 (0.19) | −7.37 (0.25) |
| 4      | −8.16\textsuperscript{a} (0.03)\textsuperscript{S6} | −10.07 (0.20) | −10.61 (0.21) | −11.77 (0.19) | −11.54 (0.25) | −10.89 (0.17) | −10.93 (0.20) |
| 5      | −8.99\textsuperscript{a} (0.07)\textsuperscript{S7} | −8.89 (0.17) | −7.71 (0.25) | −9.76 (0.30) | −10.10 (0.19) | −9.79 (0.18) | −10.00 (0.20) |
| 6      | −9.45\textsuperscript{b} (0.13)\textsuperscript{S8} | −8.16 (0.20) | −7.21 (0.14) | −9.27 (0.26) | −9.61 (0.17) | −9.98 (0.21) | −9.51 (0.24) |
| 7      | −10.41\textsuperscript{b} (0.23)\textsuperscript{S9} | −9.11 (0.17) | −9.43 (0.16) | −11.44 (0.26) | −11.68 (0.18) | −11.03 (0.20) | −9.75 (0.19) |

Statistics

|        | MSE   | RMSE  | y-int | slope | $r$   | $\tau$ |
|--------|-------|-------|-------|-------|-------|--------|
|        | 0.00  | 0.14  | -0.02 | 1.00  | 1.00  | 0.89   |
|        | (0.06) | (0.07) | (0.39) | (0.05) | (0.01) | (0.11) |
|        | 0.00  | 1.14  | -3.03 | 0.63  | 0.70  | 0.44   |
|        | (0.44) | (0.21) | (2.76) | (0.33) | (0.25) | (0.27) |
|        | 0.28  | 1.53  | -3.23 | 0.57  | 0.55  | 0.33   |
|        | (0.58) | (0.27) | (3.47) | (0.42) | (0.27) | (0.27) |
|        | -0.84 | 1.61  | -0.30 | 1.07  | 0.74  | 0.40   |
|        | (0.56) | (0.51) | (3.74) | (0.45) | (0.21) | (0.24) |
|        | -0.92 | 1.61  | 0.45  | 1.18  | 0.79  | 0.44   |
|        | (0.53) | (0.44) | (3.57) | (0.42) | (0.18) | (0.24) |
|        | -0.76 | 1.29  | 0.03  | 1.10  | 0.83  | 0.50   |
|        | (0.42) | (0.36) | (2.97) | (0.35) | (0.16) | (0.23) |
|        | -0.49 | 1.23  | -0.77 | 0.98  | 0.77  | 0.38   |
|        | (0.45) | (0.38) | (3.47) | (0.42) | (0.25) | (0.29) |
Table S10: Free energy contributions and final values for the release of the apo protein conformational restraints to the open state ($\Delta G_{rl,p}$), using the three water models. Also shown are the release free energy of the protein to the metastable closed state ($\Delta G_{rl,p,cl}$), and the value of $\Delta G_{conf,p}$. Uncertainties are shown in parenthesis.

|        | $\Delta G_{conf,p}$ | $\Delta G_{conf,at}$ | $\Delta G_{conf,pull}$ | $\Delta G_{conf,rl}$ | $\Delta G_{rl,p}$ | $\Delta G_{rl,p,cl}$ |
|--------|----------------------|-----------------------|------------------------|----------------------|-------------------|---------------------|
| TIP3P  | -2.54 (0.12)         | 11.78 (0.02)          | -1.01 (0.09)           | -14.74 (0.07)        | -3.97 (0.12)      | -1.43 (0.02)        |
| TIP4PEw| -3.72 (0.18)         | 11.97 (0.05)          | -2.86 (0.15)           | -14.58 (0.07)        | -5.47 (0.17)      | -1.75 (0.03)        |
| SPC/E  | -1.67 (0.21)         | 11.75 (0.04)          | -0.28 (0.19)           | -14.87 (0.07)        | -3.40 (0.21)      | -1.73 (0.03)        |
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