Protein complex formation by acetylcholinesterase and the neurotoxin fasciculin-2 appears to involve an induced-fit mechanism

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Specific, rapid association of protein complexes is essential for all forms of cellular existence. The initial association of two molecules in diffusion-controlled reactions is often influenced by the electrostatic potential. Yet, the detailed binding mechanisms of proteins highly depend on the particular system. A complete protein complex formation pathway has been delineated by using structural information sampled over the course of the transformation reaction. The pathway begins at an encounter complex that is formed by one of the apo forms of neurotoxin fasciculin-2 (FAS2) and its high-affinity binding protein, acetylcholinesterase (AChE), followed by rapid conformational rearrangements into an intermediate complex that subsequently converts to the final complex as observed in crystal structures. Formation of the intermediate complex has also been independently captured in a separate 20-ns molecular dynamics simulation of the encounter complex. Conformational transitions between the apo and liganded states of FAS2 in the presence and absence of AChE are described in terms of their relative free energy profiles that link these two states. The transitions of FAS2 after binding to AChE are significantly faster than in the absence of AChE; the energy barrier between the two conformational states is reduced by half. Conformational rearrangements of FAS2 to the final liganded form not only bring the FAS2/AChE complex to lower energy states, but by controlling transient motions that lead to opening or closing one of the alternative passages to the active site of the enzyme also maximize the ligand's inhibition of the enzyme.

conformational transitions | protein–protein binding

Maintaining effective molecular recognition between interacting proteins is of fundamental importance for many biological processes, such as signal transduction, cell regulation, the immune response, the assembly of cellular components, and regulation of enzymatic activities. Recognition between substrates and enzymes, the earliest model for selective and precise interactions, was originally explained by using the “lock and key” concept, which was introduced by Emil Fischer in the late 19th century. However, the first crystal structure of myoglobin (1) provided no obvious mechanism for the diffusion of oxygen to the heme group at the center of the protein. The induced-fit model, which was first described by Koshland (2), has since become an important concept in explaining the roles of protein flexibility in substrate binding. With recent advances in methodologies and technologies, the energy landscape of proteins (3) can be mapped out and proteins can be captured in different states (4). The preexisting equilibrium model has been introduced along with the energy landscape theory in protein folding (5, 6) to provide yet another important view of recognition and binding. These binding mechanisms can be summarized by using the thermodynamic cycle in Fig. 1. The preexisting equilibrium mechanism for binding is described by reactions 1 and 2 in Fig. 1. In reaction 1, the native state of the protein B exists in an ensemble of conformations; among these, the active form B* is significantly populated in equilibrium with other B conforma-

Fig. 1. Thermodynamic cycle for AB* complex formation reactions. A and B molecules can be considered as any pair of interacting molecules.

tions. The active B* conformer will bind selectively to molecule A to form the final complex. An alternative to this model is an induced-fit mechanism, which is illustrated in reactions 3 and 4 (Fig. 1). Reaction 3 describes formation of an encounter complex between molecules A and B; then, in reaction 4, B is changed into conformer B*, which forms the final complex, AB*. The mechanisms outlined in these models have been demonstrated in a number of experiments (4, 7, 8). These concepts should, therefore, be useful in characterizing mechanisms of protein–protein binding, and because of the prevalence of specific, rapid interactions between proteins, should prove relevant to understanding biological processes. In conjunction with our previous molecular dynamics (MD) studies (9, 10) of the neurotoxin fasciculin-2 (FAS2) and acetylcholinesterase (AChE), the above binding mechanisms are used to describe the formation of FAS2/AChE complexes as seen in crystal structures (11, 12).

FAS2 (13) is a neurotoxin from green mamba snake venom and a potent inhibitor of AChE, the enzyme that regulates nerve impulses at cholinergic synapses by rapidly catalyzing the hydrolysis of the neurotransmitter, acetylcholine. This system is especially suitable for demonstrating molecular binding mechanisms because not only does FAS2 bind to AChE with a very high affinity (10^{-12} M), but it also does so at near the diffusion-controlled limit (10^8 M⁻¹s⁻¹) (14). The previous submicrosecond MD simulations of FAS2 (9) also showed that the predominant forms of FAS2 in solution are different from the liganded

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Abbreviations: MD, molecular dynamics; TMD, targeted MD; FAS2, fasciculin-2; AChE, acetylcholinesterase; RMSD, rms distances.

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Fig. 2. The free energy profiles for conformational conversions between FAS2a and FAS2b states in solution. Black circles represent average free energies with SEM for the conversions between FAS2a and FAS2b states, and those of the reversed direction, from FAS2b to FAS2a trajectories are gray triangles. Solid lines are polynomial fittings to the fifth order.

forms observed in crystal structures (11, 12) of the FAS2/AChE complex. In the unbound conformation, FAS2:T9 (near the tip of loop I) packs against a hydrophobic pocket that is formed by FAS2:Y4, FAS2:A12, FAS2:R37, and FAS2:Y61 (see Fig. 6, which is published as supporting information on the PNAS web site). To keep the notation consistent with the previous study, we designate this conformation as FAS2a and the liganded form as FAS2b. The topology of the loop I of the liganded form has FAS2:T9 extended into solution, whereas the hydrophobic pocket is occupied either by an aliphatic chain of the β -octyl-glucoside molecule [the detergent that was cocrystallized in the apo-FAS2 structures (13)] or the hydrophobic side chain of V73 of AChE as seen in the FAS2-AChE complex (11, 12). In addition, the encounter complex was found to be stable enough to allow conformational switching to occur. These considerations lead to the following question: does the formation of the final FAS2/AChE complex occur by the preexisting equilibrium-binding mechanism or the induced-fit mechanism? In this study, conformational transitions of FAS2 in the presence and absence of AChE are described in terms of their relative free energy profiles that link the two conformational states. To gain further insight into the inhibition mechanisms, the dynamical transitions between these two states have also been characterized by analyzing multiple targeted MD (TMD) trajectories and by comparison to the previous submicrosecond dynamics of apo-FAS2 (9) and the dynamics of AChE (10).

Results and Discussion

Conformational Conversions of FAS2 in Solution. The previous study indicated that the distance, q , from the tip of loop I, FAS2:T9, to the C terminus, FAS2:Y61, is an excellent choice for a reaction coordinate. Rms distances (RMSD) of each conformation with FAS2b as reference structure are highly correlated with the distance q in depicting the progress of conversion from the FAS2a to FAS2b conformers, and the corresponding trend can also be observed in the reverse direction. Thus, using this reaction coordinate q , the average free energy of configurations with bin widths $\Delta q = 1.0 \text{ \AA}$ is calculated for all 10 TMD trajectories by using Eq. 3 in *Methods*. Fig. 2 depicts the average free energy profile along the reaction coordinate, q . Interestingly, TMD trajectories using FAS2a as reference structures yield a very similar energy profile to those that have FAS2b as reference structures. As can also be seen in Fig. 2, conformations in FAS2a states have lower energy than those in FAS2b states. It is of interest to note that FAS2a is a stable and predominant state in solution, consistent with the previous study (9). The energy barrier between the two states is remarkably high. It is possible that this barrier might be somewhat smaller because of

partial denaturation in the transition state (15, 16). Such a high energy barrier still leads to a slow conformational conversion between FAS2a and FAS2b in solution. It seems unlikely that there would be frequent sampling of the two conformational populations in equilibrium, as suggested for the preexisting equilibrium concept, which is based on the rugged energy landscape with thermal barriers of a few $k_B T$ s (3). This slow conversion raises the interesting question of how FAS2 changes its conformations when it is complexed with AChE.

Conformational Conversions of FAS2 Bound to AChE. The progress of conversions between FAS2a and FAS2b in complex with AChE can also be described by using the distance q as the reaction coordinate (Fig. 7, which is published as supporting information on the PNAS web site). As can be seen from this plot, the RMSD is again highly correlated with q ; this distance increases when FAS2a converts to FAS2b conformers. The reverse direction from FAS2b to FAS2a also has a high correlation, that is, q decreases as FAS2 adopts FAS2a conformations. Interestingly, all TMD trajectories appear to sample conformations with $q \approx 8\text{--}10 \text{ \AA}$ more frequently. A careful analysis of an average structure of snapshots with q in a range of $9\text{--}10 \text{ \AA}$ shows that the FAS2:T9 side chain no longer packs against the hydrophobic pocket, as other hydrophobic residues such as AChE:V73 compete for the pocket (9, 11, 12). AChE:P78 at the tip of the long omega loop (AChE:C69-C96) also moves toward the hydrophobic pocket from a distance of $\approx 10 \text{ \AA}$ (measuring from the AChE:P78CG and FAS2:A12CA) to $\approx 5 \text{ \AA}$ for the FAS2a/AChE and FAS2b/AChE conformations, respectively (see Fig. 8, which is published as supporting information on the PNAS web site). As a result, the FAS2:T9 side chain no longer packs against the pocket. It moves out and forms a hydrogen bond between the hydroxyl group of FAS2:T9 and the carbonyl oxygen atom of AChE:D74 of the long omega loop. This hydrogen bond remains intact for all structures with q values of $9\text{--}10 \text{ \AA}$. We designated this conformation as a FAS2i/AChE intermediate, in the sense that it is between the initial encounter complex and the final FAS2b/AChE conformer. It is notable in this regard that the 20-ns MD trajectory of the encounter complex also relaxes to the FAS2/AChE intermediate structures within a 1.5-ns period (Fig. 9, which is published as supporting information on the PNAS web site). In Fig. 8, three distinct conformers of FAS2 in complex with AChE are rendered: a FAS2a/AChE ($q \approx 5 \text{ \AA}$), an intermediate structure of FAS2/AChE ($q \approx 10 \text{ \AA}$), and FAS2b/AChE ($q \approx 20 \text{ \AA}$). Significant backbone displacements of the long omega loops for these complexes, particularly for the outer portion of the omega loop, are depicted in addition to the inward motions of the tip of the omega loop, AChE:P78. Enhanced mobility of the outer portion of the omega loop has also been observed in previous studies (10, 17, 18) using fluorescence anisotropies and MD to probe dynamics of this omega loop of the complex, particularly AChE:E81 and AChE:E84.

To investigate how stable the FAS2i/AChE structures are in comparison to other conformations along q , the free energy profiles of the conformational transitions between FAS2a and FAS2b in complex with AChE, and in the reverse direction, are plotted as functions of the progress variable, q (Fig. 3A). Surprisingly, there is a local minimum $\approx 7\text{--}8 \text{ \AA}$, indicating that intermediate structures with an average q value of $7\text{--}8 \text{ \AA}$ are more stable than the initial encounter complex that is formed when FAS2a (q value of $5 \text{ \AA}$) rapidly binds to AChE. Therefore, conformational rearrangements from the initial encounter complex to the FAS2i/AChE are energetically favorable. That the FAS2a/AChE complex moves down in free energy to stable subconformers is also demonstrated from the 20-ns MD trajectory of FAS2a/AChE complex (Fig. 9). The probability distribution of q values shifts from an average q value $\approx 5 \text{ \AA}$ (for the 150-ns FAS2a MD trajectory) to the value $\approx 10 \text{ \AA}$ (for the 20-ns

Fig. 3. The free energy profiles of FAS2 conformational conversions in the presence of AChE. (A) Black circles represent average free energies and SEM for the conversions between FAS2a and FAS2b states in complex with AChE; those of the reversed direction, from FAS2b to FAS2b, are gray. (B) Energy profiles of conversions from FAS2a to FAS2b in presence of AChE (black line) is compared with the profile in the absence of AChE (gray line).

FAS2a/AChE MD trajectory) as can be seen in Fig. 10, which is published as supporting information on the PNAS web site. Moreover, experimental stop-flow measurements of FAS2/AChE complex formation show biphasic kinetics, which is an indication of conformational rearrangements of the complex, and perhaps the formation of intermediate structures (ref. 9 and Z. Radic, personal communication).

The energy barrier between FAS2a and FAS2b is significantly lower for the conversions in the presence of AChE than without AChE; namely, it is reduced by half (Fig. 3B). Although much less strain is expected than in the absence of AChE, some additional reduction of the barrier caused by local denaturation is possible (15, 16). The final complex of FAS2b/AChE as seen in crystal structures is the most stable state (Fig. 3), which is consistent with the fact that FAS2 facilitates crystallization of FAS2/AChE complex by shifting the complex structures toward more stable states (11, 12). From the free energy profiles for conformational conversions, unbound FAS2a forms are much more stable than those of FAS2b, which is consistent with the previous studies showing that the predominant unliganded conformations are FAS2a. The energy barrier between these two states might be very high, which leads to conformational conversions between these states taking longer than the lifetime of the encounter complex (≈ 1 ns). Such features suggest that FAS2a conformers quickly bind to AChE forming the initial encounter complex; the complex then undergoes “catalyzed” conformational rearrangements to stable intermediate structures, and subsequently, to the final complex as seen in crystal structures. This mechanism is summarized in Figs. 3 and 8, which demonstrate structural changes over the course of complex formation (an animation of the final complex formations is shown in Movie 1, which is published as supporting information on the PNAS web site).

Allosteric Inhibition Mechanism. Previous studies (11, 14, 19) have shown that FAS2 inhibits AChE by sterically blocking the main gorge entrance and allosterically disrupting the catalytic triad based near the bottom of the 20-Å deep and narrow gorge. As demonstrated above, lowering the energy barrier for conformational conversions of FAS2 when it is bound to AChE is another example of the dynamic flexibility of the enzyme, particularly the long omega loop. Its intrinsic flexibility has also been exploited in the click chemistry synthesis of femto-molar inhibitors (20), some of the strongest binding compounds known to date. This study suggests that AChE facilitates FAS2 inhibition by allowing the loop I of FAS2 to extend into the crevice near the lip of the gorge to maximize the surface area for contact of loop II at the gorge entrance and to make contact with the outer portion of the omega loop. Significant structural rearrangements of the

backbone of the long omega loop are observed, such as the tip of the loop moving toward the hydrophobic pocket of FAS2 (Fig. 8). The distance between AChE:P78CG and FAS2:A12CB is highly correlated to the conversion of FAS2a to FAS2b in complex with AChE (Fig. 4). This distance shortens, as the conversion between FAS2a and FAS2b forms progresses; the latter is indicated by increasing q values. Mobility of the tip of the long omega loop, which consists of residues that make up part of the main gorge entrance, allows AChE to make stronger contacts with FAS2. Sterically, this motion leads to the complete blocking of the main gorge entrance. The distances between AChE:D74 and FAS2:T9 also show correlations with the formation of the stable intermediates and the progress of the conformational conversion between FAS2a and FAS2b (Fig. 4). Moreover, the 20-ns MD trajectory of FAS2a/AChE was observed to convert to the intermediate structure with an average q value ≈ 10 Å and with a strong hydrogen bond between AChE:D74 and FAS2:T9 (data not shown). Only after losing contacts with this residue does a significant conformational change occur (Fig. 4). AChE:D74, near the main gorge entrance, has been shown to play important roles in substrate binding at the peripheral site (21, 22).

It is of interest to point out that the 20-ns MD trajectory of FAS2a/AChE was initiated from a model structure that has FAS2a bound to AChE with a closed back-door passage [formed by AChE:W86, AChE:Y448, AChE:G449, and AChE:V451, an alternative channel leading to the active site of AChE (10, 19, 23)]. To detect the openings of this passage, a probe with the same radius as a water molecule was rolled around the surface of these residues; the back door is judged to be open if the solvent-accessible surface is continuous from the active site

Fig. 4. Conformational conversion correlations. Shown are the correlations of the reaction coordinate q to H-bond formation, i.e., the distance between AChE:D74O and FAS2:T9OG (black line), and to hydrophobic displacement, i.e., the distance between AChE:P78CG and FAS2:A12CB (gray line).

A

B

Fig. 5. The electrostatic potential mapped on the solvent-accessible molecular surface for the FAS2a/AChE conformer with the back door open to a radius >2.0 Å (A) and the FAS2b/AChE conformer with the back door closed to a radius <1.4 Å (B). The color scheme is the same for both images: $+1 k_B T/e$ (blue) and $-8 k_B T/e$ (red).

marked by AChE:E202 or S203 to the enzyme surface at this back-door passage (Fig. 11, which is published as supporting information on the PNAS web site). Surprisingly, the back-door passage of this FAS2a/AChE trajectory is open most of time, after the conformational rearrangement from the encounter complex to the intermediate. It has been suggested that enhanced opening of the back door could contribute to the residual activity of AChE when FAS2 is bound (10, 23).

Interactions between the loop I of FAS2 and the outer portion of the long omega loop have been shown to correlate to the opening of the back door (10). As FAS2a converts to FAS2b, the side chain of FAS2:R11 of the FAS2a conformer (which mimics loop I of FAS2b conformers in binding to the crevice behind the gorge entrance), swings out to form strong salt-bridge interactions with either AChE:E84 or AChE:E91 (Fig. 12, which is published as supporting information on the PNAS web site). Motions of the long omega loop of AChE, which are enhanced when FAS2 is bound to AChE, have also been shown in the previous MD and fluorescence anisotropy studies (17, 24). Opening of the back door is highly correlated to the salt-bridge formation between FAS2:R11 and AChE:E91 (10). The outer portion of the omega loop exhibits considerable changes in its secondary structures such as a formation of helical segment AChE:E81-M85 (Fig. 12). As can also be seen in Fig. 12, rotation of the AChE:W86 side chain is allowed as changes in backbone motions of the outer portions of the omega loop create cavities near the ring of AChE:W86. This motion is reminiscent of other transient packing defects and gating effects (25). These collective motions effectively control access to the active site via the back door and facilitate the conversions of FAS2a to FAS2b. Based on mapping the electrostatic potential onto the 1.4-Å solvent-accessible molecular surface, there are strong negative electrostatic potentials present around the back door for both closed and open conformers (Fig. 5). Residual activity for the FAS2-bound enzyme has been observed in experimental studies (14, 26, 27). It seems possible that when the main gorge entrance is sealed off the charge distribution of the back door creates an

electric field, guiding substrates to the active site via this opening.

To explore further how the formation of the final complex contributes to inhibition, the binding free energies of FAS2/AChE complex are computed and tabulated in Table 1 (see also Table 2, which is published as supporting information on the PNAS web site). FAS2a-bound AChE, both closed and open back-door conformers, are less favorable than FAS2b-bound AChE with the closed back-door conformer, which has the best free energy of binding (-37 kcal/mol), which is consistent with the notion of maximizing inhibition. As also noted in the previous study, the estimated values from the molecular modeling are overestimated in comparison to the binding energy calculated from the equilibrium constants for FAS2 binding to AChE by Radic *et al.* (14). The binding free energies obtained here do not incorporate certain entropic contributions associated with the protein-protein binding, so that the estimations are not inconsistent with the experimental measurements. For FAS2a-bound AChE, AChE conformers with open back doors are more stable than those with closed back doors; the opposite is observed when FAS2b is in a complex with AChE, in part because AChE conformers with the closed back doors are more stable than those with the open back doors (Table 1). Electrostatic calculations for the enzyme and electron density maps for crystal structures suggest that the presence of a small cation near the active site is required for correct enzyme function (28–30). In closed back-door conformations, the indole ring of

Table 1. Estimates of free energy of binding (kcal/mol)

Complex	G_{FAS2}	G_{AChE}	$G_{\text{FAS2/AChE}}$	$\Delta G_{\text{FAS2/AChE}}$
FAS2a/AChE _{closed}	-2,772	-21,919	-24,699	-8.0
FAS2a/AChE _{open}	-2,737	-21,935	-24,701	-29.0
FAS2b/AChE _{closed}	-2,764	-22,131	-24,932	-37.0
FAS2b/AChE _{open}	-2,718	-21,985	-24,727	-24.0

The free energy, $G_{\text{MMPBSA}} = U_{\text{MM}} + W_{\text{PB}} + W_{\text{NP}}$ is computed for each conformer. The binding energy, $\Delta G_{\text{FAS2/AChE}} = G_{\text{FAS2/AChE}} - G_{\text{AChE}} - G_{\text{FAS2}}$.

AChE:W86 is within proximity of cation- π interactions (Fig. 12). Favorable cation- π interactions are among the factors that may contribute to closed back-door conformers being more stable than the open forms.

Conclusion

Conformational changes are vitally important in many aspects of protein function. The present study reveals the involvement of such changes in both proteins in the formation of a protein-protein complex. The intrinsic flexibility of AChE, particularly of the long omega loop, facilitates rapid conversions from FAS2a to FAS2b in the inhibition process. The initial encounter complex formed by FAS2a and AChE is stable enough to allow switching to the final complex that is observed in crystal structures. Free energy profiles for the process of complex formation point to an induced-fit mechanism rather than a preequilibrium mechanism in the present case.

Methods

Preparation of Reference Structures for TMD Simulations. FAS2, a 61-residue peptide with four disulfide bonds, is a member of the three-finger toxin family (13) (see Fig. 6). Members of this family have a similar three-finger fold topology, but they have a variety of biological activities such that some members can bind other targets. For example, α neurotoxins are antagonists of the nicotinic acetylcholine receptor, and manbin is an antagonist of platelet aggregation and cell-cell adhesion (13). Two major conformational states of FAS2 were identified in the previous MD simulations (9), namely unbound and liganded forms. Ten snapshots of each submicrosecond MD trajectory of apo-FAS2a and apo-FAS2b were chosen as reference structures for TMD simulations (9). That study also showed that the encounter complex of FAS2a/AChE is reasonably stable, relative to the separated proteins. The same encounter complex was used as a starting structure for the MD simulation of FAS2a/AChE. This FAS2a/AChE encounter complex was obtained by docking of the FAS2a conformer to snapshots of the 15-ns MD apo-AChE trajectory. The complex structure was then relaxed to optimize favorable interactions between the apo-FAS2 conformer and AChE. The final configuration of the complex is one in which loops II and III of the liganded FAS2a fit well to the crystallographic structure of the liganded FAS2b, as observed in the crystallographic structures of FAS2b/AChE. Six sodium ions were then added to neutralize the system, and it was solvated with 35,796 TIP3P water molecules. A standard MD procedure was performed beginning with 1,000 steepest-descent energy minimization steps until the system was significantly relaxed. Velocities were reassigned from 300-K Maxwellian distributions every 1 ps for 100 ps, and the system was then equilibrated for 1,000 ps. The simulation was conducted by using the isobaric-isothermal ensemble (31) at 300 K and 1 atmosphere and using long-range non-bonded interactions with a 12-Å residue-based cutoff. Long-range electrostatic forces were calculated by using the particle-mesh Ewald method (32) in which a direct sum was evaluated explicitly with a cutoff of 12.0 Å and charges were interpolated to a grid of 1-Å resolution by using the B-spline fourth-order function. After equilibration, a 20-ns MD trajectory for the FAS2a/AChE complex was collected. Ten snapshots, which are designated as encounter complex conformers having the distance between FAS2:T9 and FAS2:Y61 of ≈ 5.0 Å (see *Results and Discussion* for the choice of a reaction coordinate), were chosen as reference structures for the TMD simulations of FAS2 in complex with AChE.

TMD Trajectories in the Presence and Absence of AChE. Important protein motions often occur in time scales (microseconds to milliseconds) that have not yet been reached for all-atom MD simulations of biologically relevant size. Many enhanced sampling methods [hyperdynamics and accelerated MD, replica

exchange MD, steered MD, and TMD; see the review article by Adcock and McCammon (33)] and coarse-grain modeling (elastic network and Gō-like models) (34) have increasingly become powerful tools allowing simulations of such systems. In this study, TMD (35, 36), in which only mass-weighted RMSD of loop I residues of FAS2a conformers to those of FAS2b conformers were restrained by a harmonic force constant, k , were used to sample conformational transitions between FAS2a and FAS2b states in the presence and absence of AChE. The biased simulations were conducted by using a potential energy function that is modified by a harmonic restraint potential, $U_{\text{res}}(X) = \frac{1}{2}k(\text{RMSD}_f - \text{RMSD}_i)^2$. RMSD_f is the RMSD of the final conformation, and RMSD_i starts with the value equal to the RMSD between the initial and final structures. The rest of the atoms, including solvent molecules, were not restrained. All TMD trajectories were obtained by using the ITGTMD AMBER 8 module (37). The same MD parameters as described in the MD setup were used to ensure that the systems behave under the same standard conditions. A total of 20 TMD simulations (10 with FAS2a as reference structures and 10 with FAS2b as the targeted conformations) were obtained in the absence of AChE. In the presence of AChE, 10 TMD trajectories were integrated with the FAS2b/AChE complex references and 10 TMD trajectories had the FAS2a/AChE as references.

Free Energy Profiles. For a chosen reaction coordinate q , a probability distribution function, $P(q)$, along this reaction coordinate q is defined by

$$P(q) = \frac{\int d\Omega' \delta(q' - q) e^{-\beta V(\Omega')}}{\int d\Omega' e^{-\beta V(\Omega')}}, \quad [1]$$

where $\beta = (k_B T)^{-1}$, Ω' represents a particular set of the coordinates of all of the atoms in the system, $V(\Omega')$ is the corresponding energy, and q' is the value of the reaction coordinate. The delta function $\delta(q' - q)$ equals zero for $q' \neq q$, so that the probability, $P(q)$ at $q' = q$ is a Boltzmann probability. Thus, the free energy profile in terms of the natural log of $P(q)$ is used to define the potential of mean force,

$$G(q) = -kT \ln P(q). \quad [2]$$

This free energy evaluation is obtained by using the post-MD free energy process known as the Molecular Mechanics Poisson-Boltzmann Solvent Accessible (MMPBSA) surface method (38).

$$G_q = \langle U_{\text{MM}} \rangle_q + \langle \Delta W_{\text{sol}} \rangle_q, \quad [3]$$

where $\langle \rangle_q$ denotes an average over the conformers in each bin width along the chosen reaction coordinate, q . The internal energy, U_{MM} , of the solute was calculated as its energy in the gas phase. The solvation free energy ΔW_{sol} is written as a sum of the hydrophobic energy, ΔW_{np} , and the electrostatic solvation energy, ΔW_{PB} . The solute hydrophobic energy was calculated as the sum of products of solvent-accessible surface area (SASA) by the atomic solvation parameters, $\Delta W_{\text{np}} = \gamma \times \text{SASA} + \beta$, where $\gamma = 0.00542 \text{ kcal/mol } \text{\AA}^2$ and $\beta = 0.92 \text{ kcal/mol}$ (38, 39). The SASA was calculated by using the MSMS 2.5.3 program (40) with a water probe radius equal to 1.4 Å. The electrostatic contribution was computed by using the adaptive Poisson-Boltzmann solver (41). The Poisson-Boltzmann calculations were run with a temperature of 300 K, a solvent probe radius of 1.4 Å, a solvent dielectric constant of 78.4, and a reference gas phase dielectric

constant of 1.0. The dielectric constant of the protein's interior was set at 1.0 to be consistent with the MD simulation setup.

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