A conserved protonation-dependent switch controls
drug binding in the Abl kinase

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In many protein kinases, a characteristic conformational change (the "DFG flip") connects catalytically active and inactive conformations. Many kinase inhibitors—including the cancer drug imatinib—selectively target a specific DFG conformation, but the function and mechanism of the flip remain unclear. Using long molecular dynamics simulations of the Abl kinase, we visualized the DFG flip in atomic-level detail and formulated an energetic model predicting that protonation of the DFG aspartate controls the flip. Consistent with our model's predictions, we demonstrated experimentally that the kinetics of imatinib binding to Abl kinase have a pH dependence that disappears when the DFG aspartate is mutated. Our model suggests a possible explanation for the high degree of conservation of the DFG motif: that the flip, modulated by electrostatic changes inherent to the catalytic cycle, allows the kinase to access flexible conformations facilitating nucleotide binding and release.

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of human c-Abl containing only the kinase domain. (Because the kinase domains of c-Abl and BCR-Abl are identical in sequence, we refer to this common domain below as simply “Abl.”) Our analysis of these simulations allowed us to formulate a model of key dynamic, energetic, and structural factors involved in DFG flips. To validate our model experimentally, we used fluorescence assays of Abl–imatinib binding to probe DFG conformation, the results of which confirmed our central computational findings.

Like other eukaryotic protein kinase domains, Abl consists of a smaller N-terminal lobe and a larger C-terminal lobe (the N-lobe and the C-lobe, respectively), with the ATP-binding site located between them. The N-lobe is composed of 5 β-strands and a helix referred to as αC, whereas the C-lobe is predominantly helical. The DFG motif borders the ATP-binding site and is located between the 2 lobes, at the N terminus of a flexible segment known as the activation loop. Fig. 1A and C shows Abl adopting DFG-in (22) and DFG-out (7) conformations, respectively, whereas Fig. 1B shows an important intermediate conformation observed in our simulations of the DFG flip. This conformation differs from the other 2 in that helix αC is displaced away from the binding site (the αC-out conformation, by contrast with the αC-in conformation shown in Fig. 1A and C). A recently solved crystal structure of Abl also adopts an αC-out conformation, which has been suggested as a potential intermediate in the DFG flip (17). Although kinases in their active states adopt αC-in conformations, many kinases in their inactive states are known to adopt αC-out conformations, first defined in Cdk (23) and Src (24, 25) kinases, but now seen in many others—including EGFR (26), whose αC-out conformation is recognized by the cancer drug lapatinib. Thus, just as many kinases can access alternative conformations through a DFG flip, many kinases can access alternative conformations through a transition between αC-in and αC-out conformations.

To study the conformational changes associated with the DFG flip without introducing any biasing forces in our MD simulations, we “loosened” helix αC by mutating to alanine a methionine residue that previous studies suggested might be involved in stabilizing hydrophobic interactions between helix αC and the remainder of the protein. In our simulations of the resulting protein, we were able to observe the DFG flip and its coupling with helix-αC motion at an atomic level of detail. Analysis of these simulations led us to propose an energetic model in which the protonation state of Asp-381, which is influenced by local electrostatic interactions, serves as the switch that controls the flip. On the basis of our simulation results, we made certain predictions regarding the pH dependence of Abl–imatinib binding, then performed fluorescence quenching experiments to test these predictions. The results of these experiments were consistent with our model (and also allowed us to estimate the time scale of the DFG flip to be tens of milliseconds). Our findings could be interpreted as providing mechanistic support for the suggestion (27) that the DFG flip plays a significant role in the catalytic cycle of protein kinases by facilitating nucleotide binding and release.

Results and Discussion

Simulations of Abl. We performed a total of >2 μs of all-atom MD simulations of Abl in explicit water, using the OPLS-A/A force field (28, 29) for proteins, with most individual simulations between 100 and 300 ns in length. All simulations were performed at constant temperature (300 K) and pressure (1 bar); see SI Text.

N-Pocket as Part of the DFG-Flip Pathway. Standard electrostatics calculations (30) (see SI Text) suggest that in a DFG-in structure of Abl [PDB ID code 2F4J; Young et al. (22)], the DFG aspartate has an elevated pKₐ. In a DFG-out structure of Abl [PDB ID code 1OPK (7)] the DFG aspartate appears to be protonated and hydrogen bonded to a backbone carbonyl group. These observations led us to hypothesize that the protonation of the DFG aspartate might be an important determinant of DFG conforma-

tion, and we therefore performed simulations with the residue in different protonation states. We started 4 simulations in the active DFG-in conformation, including 2 (simulations 1a and 1b in Table S1) with Asp-381 protonated and 2 with Asp-381 deprotonated (simulations 2a and 2b). No DFG flip was observed in these simulations, suggesting that its time scale is much longer than the several hundred nanoseconds we simulated. This result is consistent with NMR studies of the p38 (13) and PKA (31) kinases, which are suggestive of microsecond- to millisecond-scale DFG motions.

In all 4 simulations, however, substantial motion of helix αC was observed, despite the fact that no mutation was introduced to promote such motion. In the simulations with Asp-381 protonated, this motion was particularly pronounced, leading to the formation of the Glu-286–Arg-386 salt bridge characteristic of the αC-out conformation and emergence of a hydrophobic pocket (the N-pocket; see Fig. 1B) at the base of the N-lobe near Phe-382 (Fig. 24). The N-pocket, which is contiguous with a region exploited by many drugs (the so-called “kinase specificity pocket”), has been suggested to play a role in facilitating the DFG flip (17). Although simulations 1a and 1b showed the transition from αC-in to αC-out (Fig. 1), we did not observe Phe-382 entering into the N-pocket, apparently because of interactions between this residue and the helix-αC residue Met-290. Likewise, we did not observe Phe-382 and Met-290 to adopt the exchanged positions they assume in crystal structures of Abl and Src kinases in αC-out conformations. These residues remained, for the most part, closely packed during the simulations (Fig. S1), and when they were briefly driven apart by thermal fluctuations, Met-290 remained a steric barrier at the entrance to the N-pocket (Fig. 2A).

To lower this barrier, we attempted to further increase the extent of motion of helix αC by introducing an M290A mutation. Met-290 is not a conserved residue in protein kinases, and the c-Src mutant corresponding to M290A maintains substantial activity (Fig. S2B). In Abl, Met-290 is part of a “hydrophobic spine” that stabilizes the active conformation (32), and we anticipated that the M290A mutation might therefore facilitate entry of Phe-382 into the N-pocket by allowing a larger range of motion of helix αC, as well as by directly reducing the size of the obstructing residue. We
initiated simulations of the M290A mutant with the DFG aspartate both protonated (simulations 3 a–e) and deprotonated (4 a and b). With the M290A mutation, Phe-382 was indeed observed entering and leaving the N-pocket (Fig. 2b), with maximum residence times of 6 and 25 ns in simulations 4a and 4b, respectively. These results support the structural observation that Met-290 presents a barrier to the entry of Phe-382 into the N-pocket. Protonation of Asp-381 also appears to be a factor, because none of the simulations 3 a–e showed Phe-382 entering the N-pocket, presumably because of an indirect coupling between the (protonation-dependent) conformation of Asp-381 and the conformation of Phe-382. This failure of Phe-382 to enter the N-pocket when Asp-381 is protonated, however, may well be due to the limited simulation time scale, over which Asp-381 is trapped by a hydrogen bond (with Asp-363).

Simulations of the DFG-in to DFG-out Flip. Because the DFG-in conformation with Phe-382 in the N-pocket (Fig. 1b) may be an important intermediate of the DFG flip (17), we initiated 4 simulations of the M290A mutant from this conformation (taken from simulation 4a). In 2 simulations (5 a and b) Asp-381 was deprotonated and the DFG flip was not observed. In the other 2 simulations (6 a and b), Asp-381 was protonated and the DFG flip was observed after 68 and 76 ns, respectively. In our simulations, the M290A mutation was found to be required for Phe-382 to enter the N-pocket, but once this conformation had been reached, the DFG flip occurred even in the absence of this mutation. This was shown in simulation 7, which started from the same conformation, with Asp-381 protonated and Ala-290 mutated back to methionine; the DFG flip was observed after 33 ns. The 3 observed DFG flips followed similar pathways, as described below.

Fig. 3 shows a sequence of simulation conformations to illustrate the DFG flip. The motion of the 2 side chains through the crowded environment resembles a crankshaft motion around the main chain, and the DFG main chain also undergoes conformational rearrangement as an integral part of the flip (Fig. 3B).

The simulations indicate that large-scale motions play a key role in enabling the bulky side chain of Phe-382 to enter the ATP-binding site. Before the flip, we observe significant twisting and hinge-opening motions of the N-lobe with respect to the C-lobe, along with further outward displacement of helix αC leading to an αC-out conformation (Fig. 3). This leads to conformations in which Phe-382 has access to the ATP-binding site. After completion of the DFG flip, the N-lobe relaxes toward the autoinhibited conformation, and the interlobe motion subsides (Fig. S3).

A more detailed picture of the local changes occurring during the simulated DFG flip emerges from the examination of certain key dihedral angles. Several angles are different in the active and autoinhibited conformations (Fig. S4B). Protonation of Asp-381 at the beginning of the simulation causes this residue to reposition toward the C-lobe within the ATP-binding site, which is reflected in a change of its ϕ angle. There is subsequently significant motion, including large fluctuations in the ϕ angle of Gly-383 and concerted changes in most DFG main-chain dihedral angles (see Fig. S3). Until the DFG flip occurs, however, Asp-381 remains in the ATP-binding site and Phe-382 remains in the N-pocket, despite the main chain sometimes adopting DFG-out-like conformations. When the DFG motif finally flips, it is through a similar concerted change in main-chain conformation coupled with Phe-382 swinging into the ATP-binding site by means of a simultaneous change in its χ1 angle. The flip itself lasts ≈2 ns. Subsequently, the dihedral angles in the DFG motif exhibit much less variation. Such a transition between relatively flexible and rigid states is observed in all 3 simulations in which we saw DFG flips.

Structural details of the DFG flip emerging from our simulations are consistent with existing crystal structures. Analysis of a representative set of kinase crystal structures (33) (see SI Text) shows that they and the simulation structures occupy similar regions of DFG conformational space (Fig. S4B). In both crystal structures and simulations, Asp-381 is always positioned toward the C-lobe side and Phe-382 toward the N-lobe whenever the DFG motif adopts an intermediate conformation between DFG-in and DFG-out. When ordered appropriately, the X-ray structures can even provide an “animation” of a DFG flip resembling the trajectory of the simulated conformational change (Fig. S4A).

DFG Conformation Is Controlled by a Protonation-Dependent Energetic Switch. We now construct a model of the relative free energy of the DFG-in and DFG-out conformations of Abl that can be used to estimate the effects of pH changes on DFG conformation. Although MD simulation could, in principle, be used to approximate such free energies, the aggregate amount of simulation time required to obtain accurate computational estimates using currently available techniques and technologies made this approach infeasible for use in this context. Instead, our model approximately accounts for the factors contributing to each conformation’s stability by using empirical hydrophobicity data and a pKc calculation on a static structure.

The most important structural factor embodied in our model is the change in environment of residues Asp-381 and Phe-382 caused by a DFG flip. In the DFG-in conformation, Asp-381 is located among charged and polar residues in the aqueous environment of the ATP-binding site, and Phe-382 is located in a hydrophobic environment. Conversely, after the DFG flip, Phe-382 is in the polar environment of the ATP-binding site, and Asp-381 is in a mostly hydrophobic environment. Because there is a very high free-energy cost to bury a charge in a hydrophobic environment (34), we assume DFG-out conformations to have Asp-381 protonated. Because our simulations point to the importance of the protonation state of this residue, our model contains 3 states: (i) DFG-in, Asp-381 deprotonated; (ii) DFG-in, Asp-381 protonated; and (iii) DFG-out, Asp-381 protonated. At thermodynamic equilibrium at temperature T, the relative population of the DFG-out conformation decreases with increasing pH as (see SI Text)
$P_{\text{out}} = \frac{1}{1 + \exp(\Delta G_{2\rightarrow3}/RT) + \exp(\Delta G_{2\rightarrow3}/RT + [pH - pK_{a,in}] \times \log_{10})}$

where $\Delta G_{2\rightarrow3}$ is the free-energy cost of the DFG flip from DFG-in to DFG-out with Asp-381 protonated, $R$ is the gas constant, and $pK_{a,in}$ is the $pK_a$ of Asp-381 in a DFG-in conformation (i.e., it is the hypothetical $pK_a$ when DFG-out states are excluded, and thus differs from the actual $pK_a$).

At equilibrium, one might anticipate DFG-in conformations to dominate, because the aspartate and phenylalanine residues are favorably placed in environments matching their polarity. Three other factors combined, however, may tip the thermodynamic balance in favor of DFG-out conformations. First, in the DFG-out conformation of Abl, which we model on the kinase domain of c-Abl PDB ID code 1OPK (7), Asp-381 is well positioned to form a hydrogen bond with the exposed backbone carbonyl of Val-299. Second, contrary to what one might expect given the preponderance of reported kinase structures that appear in the DFG-in conformation, we find that the $\phi$-$\psi$ angles of the DFG aspartate in active Abl and in most other DFG-in kinase structures, fall within the unusual left-handed helix region of Ramachandran space (Fig. S4B), indicating a more strained main-chain configuration for DFG-in than DFG-out. Third, standard electrostatic calculations (30) suggest that Asp-381 has an elevated value of $pK_{a,in}$ in the absence of the ATP-bound magnesium ion, due in part to the influence of the conserved residues Glu-286, Asp-363, and Asn-368. Analysis of kinase crystal structures as a function of crystallization buffer pH (Fig. S4C) also suggests that an elevated $pK_{a,in}$ may occur in other kinases.

Combining standard literature estimates (35) and simulation results (see SI Text), we have estimated the contributions to $\Delta G_{2\rightarrow3}$ and find $\Delta G_{2\rightarrow3}$ $\sim$ $-1.1$ kcal/mol. The electrostatics yield $pK_{a,in}$ $\sim$ 6.6. With these estimates, Eq. 1 gives a large relative population of DFG-out conformations at $pH = 7$ that decreases rapidly around $pH \approx 6$ (Fig. 4B). Although the estimated parameters are, of course, only semiquantitative, the basic conclusion of a near-balance in the free energies of DFG-in and DFG-out conformations close to physiological $pH$ is robust to moderate changes in the parameters. Importantly, Eq. 1 shows that with a small $\Delta G_{2\rightarrow3}$, the DFG conformation becomes sensitive to changes in $pK_{a,in}$ and can thus be controlled by electrostatic changes in the ATP-binding site (we discuss the potential significance of this observation to kinase catalysis below). Similarly, our model predicts the effect of mutating Asp-381 or Phe-382 on DFG conformation; replacing Phe-382 with a less hydrophobic residue, for example, increases the relative population of DFG-out (Fig. 4B).

Our energetic model should also be applicable to other kinases, most directly if their activation loops adopt the common open conformation (22, 36) in DFG-in and DFG-out states (as do Abl and c-Abl in PDB ID codes 2F4J (22) and 1OPK (7), respectively). Other activation-loop conformations—particularly those occluding substrate binding, as in Abl-imatinib complexes such as PDB ID code 1HEP (8)—can lead to solvent exposure of both Asp-381 and Phe-382. Such structures are found to adopt DFG-out conformations. This observation is consistent with our analysis, because the key factor stabilizing DFG-in conformations (the hydrophobic packing of Phe-382) is missing from these structures.

**Probing the DFG-out Conformation by Using Abl–Imatinib Binding.**
With imatinib bound, Abl adopts a DFG-out conformation (8), suggesting that imatinib binding kinetics may provide a probe of DFG conformation. We derived the on-rate constant, $k_{on}$, of imatinib binding to Abl as a function of pH by using stopped-flow fluorescence assays. As shown in Fig. 4D, $k_{on}$ for wild-type Abl decreases by nearly an order of magnitude as pH increases from 5.5 to 7.5, in which range imatinib is predominantly neutral (37). We observed a very similar pH dependence for a c-Abl construct containing its kinase and SH2-SH3 domains (Fig. S2A). However, control experiments showed the imatinib-binding kinetics for Abl mutants D381A and D381N to have essentially no dependence on pH over the same range (Fig. 4A, B). The results for the mutants strongly suggest that the pH dependence observed for the wild type was a result of protonation at Asp-381.

Independent structural evidence supporting protonation of Asp-381 also exists. Most convincingly, the DFG-out c-Abl structure (PDB ID code 1OPK, resolution 1.8 Å, crystallized at pH 7.0) (7) shows 1 Asp-381 side-chain oxygen to be only 2.8 Å away from the Val-299 backbone carbonyl oxygen in a position that would be hard to rationalize if Asp-381 were not protonated in this structure. In addition, our analysis of the representative kinase structures (restricted to DFG-in conformations without bound ligands) shows that the distance of the DFG aspartate from the conserved lysine (Lys-271 in Abl) generally increases with decreasing pH of the crystallization buffer (Fig. S4C). This suggests that protonation of the DFG aspartate may be common in protein kinases.

The above results provide strong evidence that the $pK_a$ of Asp-381 is significantly raised above typical values for aspartate residues. Because imatinib is a DFG-out binder (8), another natural conclusion from the data is that protonation of Asp-381 favors DFG-out conformations. As a negative control, we have also performed fluorescence experiments using the DFG-in binder.
dasatinib (38, 39). It is thought that so-called DFG-in binders typically bind to both DFG-in and DFG-out conformations (13). We find only a very weak pH dependence for dasatinib binding (Fig. 4D), consistent with the interpretation that the pH dependence observed for imatinib binding is a result of a pH-dependent DFG conformation.

More evidence supporting a link between protonation and DFG conformation comes from the pH dependence of imatinib binding to the Abl mutant F382A shown in Fig. 4A. This mutant shows a decrease in the imatinib on-rate constant with increasing pH, although the decrease is less pronounced and shifted to higher pH compared with the wild type. This implies a higher pK_a for Asp-381 in the mutant, despite the fact that both phenylalanine and alanine are nonpolar and are positioned away from Asp-381, indicating that the mutation causes a conformational change and hence the elevated pK_a. As explained above, an F382A mutation is predicted to lead to a larger population of DFG-out conformations, thus raising the pK_a of Asp-381 in agreement with our experimental observations.

**Kinetic Scheme for Imatinib Binding.** In contrast to its kinetics, the thermodynamics of Abl–imatinib binding determined from isothermal titration calorimetry (ITC) do not show a clear pH dependence (see Table S2). This is consistent with the observation made above that in the Abl–imatinib complex—unlike in the apo DFG-out conformation—Asp-381 is solvent-exposed (because of rearrangement of the activation loop on binding; see Fig. S5A). To provide a quantitative explanation for the pH-dependent imatinib-binding kinetics, we propose the following kinetic scheme:

\[
\begin{align*}
    k_+ & \quad \text{Abl}^- (\text{DFG-in}) \xrightarrow{k_{-1}} \text{Abl}^+ (\text{DFG-in}) \\
    k_+ & \quad \text{Abl} (\text{DFG-out}) + \text{imatinib} \xrightarrow{k_{-2}} \text{Abl-imatinib},
\end{align*}
\]

where \(\text{Abl}^-\) and \(\text{Abl}^+\) denote Abl with and without protonation of Asp-381. For the imatinib concentrations (<12.5 \(\mu\)M) used in our experiments (discussed above), the fluorescence relaxation data fits a single-exponential decay, and the resultant relaxation rates are found to increase linearly with imatinib concentration (Fig. S6). Under these conditions, we thus assume that the Asp-381-protonation and DFG-flip steps of the kinetic scheme (the leftmost and middle steps in Eq. 2, respectively) are fast compared with imatinib binding to the DFG-out conformation (rightmost step in Eq. 2). This leads to effective 1-step kinetics with an on-rate constant proportional to the population of the DFG-out state: \(k_{\text{on}} = P_{\text{out}} k^+.\) In Fig. 4B, we show our estimates for \(P_{\text{out}}\) (Eq. 1) as a function of pH for Abl and 3 mutants. These estimates are clearly consistent with our experimental results for \(k_{\text{on}}\), supporting our interpretation that the protonation of Asp-381 increases the population of DFG-out.

At sufficiently high imatinib concentrations, the rate of imatinib binding to the DFG-out conformation should become comparable with that of the DFG flip. In this case, the kinetic scheme leads to effective 2-step kinetics. This is consistent with the observed fluorescence decay rates, \(k_{\text{obs}}\), which are found to deviate from a linear dependence on imatinib concentration (see Fig. 4C). In contrast, similar experiments for dasatinib binding showed no clear deviation from linearity (Fig. 4D). These results clearly demonstrate an intermediate step in imatinib binding, and support the interpretation that the pH dependence of imatinib binding arises from the DFG flip. Detailed analysis of the kinetic data of imatinib binding also revealed the DFG-flip time scale to be in the tens of millisecond (see SI Text), which is consistent with NMR studies of DFG dynamics (13). This relatively long time scale may reflect the fact that the DFG flip involves conformational changes of the activation loop beyond the DFG motif itself (33).

**Conservation of the DFG Motif and Its Role in Kinase Catalysis.** Although the DFG aspartate is clearly important for catalysis, the roles of the phenylalanine and glycine residues and the reason for their conservation are much less certain (40). Our results provide evidence that both of these residues may be essential to DFG conformational change. We find that the phenylalanine plays a key role in maintaining an energetic balance between the DFG-in and DFG-out states by compensating for the unfavorable DFG aspartate backbone torsion angles adopted in the DFG-in conformation. Our simulations also show that the DFG flip involves extensive backbone-torsion motions at the glycine residue, suggesting that the unique flexibility of glycine may serve to lower the kinetic barrier for the flip. This is consistent with work (41) suggesting that the DFG motif is optimized for functional dynamics rather than for structural stability, as is our finding that the DFG-out conformation can be populated under physiological conditions even in the absence of DFG-out binders.

If the DFG flip is to explain the conservation of the DFG motif, its role in kinase function must extend beyond the specific activation mechanism of c-Abl. The DFG flip has been suggested to promote ADP release (27), the rate-limiting step in kinase catalysis (42–44). Intriguingly, our results could be interpreted as hinting at a mechanistic explanation for such a proposed role. In ATP-bound kinases, the DFG aspartate interacts with a catalytically essential magnesium ion (Mg\(^{2+}\)) 2.3 Å away, which in turn coordinates the β and γ phosphates of ATP (5). Encouraged by observations on small G proteins (45, 46) showing that Mg\(^{2+}\) binding is weakened by ~3 orders of magnitude after transfer of the γ phosphate, we speculate that in kinases, this Mg\(^{2+}\) may likewise be released after phosphate transfer, thus reducing the positive charge in the immediate vicinity of the DFG aspartate. If this is the case, we predict that this change in the electrostatic environment of the DFG aspartate would lead to its protonation—either from the solvent or through proton transfer from the catalytic base Asp-363—causing the motif to flip to a DFG-out conformation and thus facilitating ADP release.

In such a scenario, nucleotide release could, in principle, be regulated by magnesium, consistent with the experimental observation that the ADP-release rate decreases with increasing Mg\(^{2+}\) concentration (44).

The DFG flip could facilitate ADP release, and potentially binding of the ATP-Mg\(^{2+}\) complex, by allowing the kinase to adopt a DFG-out conformation, which we find to be significantly more flexible in terms of interlobe motions than the DFG-in conformation (Fig. S7). This increased flexibility is consistent with the finding (32) that, in the active conformation, the DFG phenylalanine is part of the “hydrophobic spine” that lends stability to the DFG-in conformation. Although speculative, our suggestions are also consistent with kinetic data showing that at physiological Mg\(^{2+}\) concentration, a 10-ns time scale conformational change follows ATP binding and precedes ADP release (44), as well as with recent NMR titration experiments showing that upon the binding of ATP to PKA kinase, the DFG motif and nucleotide-binding loop of PKA exhibit microsecond to millisecond dynamics (31).

**Conclusion**

Using Abl as a model system, we have simulated the DFG flip, a conformational change known to be significant in a diverse set of kinases and, in particular, to be an important switch for c-Abl activation (7). The simulations highlight the role of large-scale conformational changes in facilitating the DFG flip and point to the importance of the protonation of the DFG aspartate residue. This conclusion is supported experimentally by our Abl-imatinib binding assays, which reveal markedly pH-dependent binding.
kinetics. Our analysis shows that the DFG conformation is determined by a number of opposing factors that approximately balance, resulting in a DFG conformation sensitive to small changes in the electrostatics of the ATP-binding site. Combined with existing structural and kinetic data, this leads us to suggest that the conservation of the DFG motif stems from the DFG flip, which switches the kinase from the less-flexible DFG-in form that the conservation of the DFG motif stems from the DFG flip, changes in the electrostatics of the ATP-binding site. Combined balance, resulting in a DFG conformation sensitive to small determined by a number of opposing factors that approximately kinetics. Our analysis shows that the DFG conformation is

Materials and Methods

Additional details are provided in SI Text.

Simulation Details. All simulations used the simple point charge (SPC) model for water (47), the standard OPLS-AA parameters for ions (48, 49), and the OPLS-AA/L force field for proteins (28, 29) as provided by the program IMPACT (50) (note that the relevant force-field option, referred to as OPLS 2003 in the IMPACT paper, has subsequently been renamed as OPLS-2005 in the actual program). All molecular dynamics simulations were performed by using the parallel MD program Desmond (19) on 64 or 128 dual-processor Opteron nodes connected by a high-speed infiniband network (Topspin, San Jose, CA). Each simulation is represented by a code number that is given, along with other key simulation details, in Table S1.

Kinetic Measurements of Drug Binding. Wild-type human c-Abl kinase domain (residues 248–532) and mutant proteins were generated and purified as described previously (51). The drug-binding kinetics is measured by monitoring the decrease of protein fluorescence at 350 nm upon excitation at 290 nm on a HORIBA Jobin Yvon FluoroMax-3 spectrophotometer for 10–20 half-lives of the transient, recorded by 1,000–2,000 data points.

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Supporting Information

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SI Text

Derivation of the Energetic Model. The energetic model contains 3 states in thermodynamic equilibrium: (i) DFG-in, Asp-381 deprotonated; (ii) DFG-in, Asp-381 protonated; and (iii) DFG-out, Asp-381 protonated. At equilibrium, the relative population of the DFG-out conformation is then

\[ P_{\text{out}} = \frac{1}{1 + \exp(\Delta G_{2\rightarrow3}/RT)} \]

where \( \Delta G_{2\rightarrow3} = G_2 - G_3 \) is the free-energy difference between states (i) and (iii), and (ii) and (iii), respectively.

Estimation of \( \Delta G_{\phi\theta} \). \( \Delta G_{\phi\theta} \) was estimated using replica-exchange molecular dynamics (REMD) (5) simulations of solvated ADFG tetrapeptide (the DFG motif is preceded by an alanine residue in Abl). The REMD simulations were performed using Desmond with force field and simulation parameters as described above; apart from the use of constant volume. The initial peptide conformation of the simulation was DFG-out, and obtained from a simulation of Abl which started in the conformation adopted by the kinase domain of PDB ID code 1OPK. Throughout the REMD simulation the N-Ca bond of the alanine and the Cα-C bond of the glycine were fixed in space to restrict the DFG backbone to conformations similar to those accessible in the full kinase domain. The simulation system is a 27 × 27 × 27 Å³ cube. Eight replicas (respectively at temperature of 300 K, 375 K, 450 K, 525 K, 600 K, 700 K, 800 K, and 900 K) were simulated for 308 ns. At a time interval of 10 ps, conformations of the DFG motif of the replica at 300 K were collected and classified into either DFG-in or DFG-out conformations based on simple geometric criteria.

Kinetic Scheme. Using the 3 different states that enter our energetic model, we propose the following kinetic scheme for imatinib binding to Abl:

\[ \text{Abl}^- (\text{DFG-in}) \rightleftharpoons \text{Abl}^\text{H}(\text{DFG-in}) \]

\[ k_{-0} \]

\[ k_{+1} \rightleftharpoons k_{-1} \]

\[ k_{+2} \]

\[ k_{-2} \]

Abl(DFG-out) + imatinib \rightleftharpoons Abl-imatinib.

The leftmost equilibrium between deprotonated and protonated forms of DFG-in is assumed to be kinetically negligible, i.e., extremely fast compared with the other steps, so that the kinetic scheme reduces to 2 steps:

\[ \text{Abl(DFG-in)} \]

\[ k_{+1} \rightleftharpoons k_{-1} \]

\[ k_{+2} \]

\[ k_{-2} \]

Abl(DFG-out) + imatinib \rightleftharpoons Abl-imatinib.

where \( k_{+1} = P_{\text{AspH}}k_{+1} \), and \( P_{\text{AspH}} = (1 + 10^{p\text{H} - p\text{K}_{\text{AspH}}})^{-1} \) is the probability in a DFG-in conformation that Asp-381 is protonated. This effective 2-step kinetic model has a fast and a slow rate constant. Assuming these have reasonably well-separated time scales, the slower rate constant, which is observed experimentally, is (6):

\[ k_{\text{obs}} = \frac{k_{+1}k_{-2} + k_{+2}k_{-1}}{k_{+1} + k_{-1} + k_{+2}L + k_{-2}} \]

where here, instead of the notation \([\text{imatinib}]\) used in the main text, we use \( L \) to denote the concentration of imatinib. Of course, for \( k_{-1} \) and \( k_{+1} \) much greater than \( k_{-2} \) and \( k_{+2} \), this reduces to

\[ k_{\text{obs}} \approx \frac{k_{+1}k_{-2}}{k_{+1} + k_{-1} + k_{+2}L} \]

To apply the model to the F382A mutant, we assume \( pK_{\text{AspH}} \) remains the same and replace \( \Delta G_{\text{pH}} = 2.8 \text{kcal/mol} \) by the KD value for alanine, \( \Delta G_{\text{Ala}} = 1.8 \text{kcal/mol} \), leading to \( \Delta G_{2\rightarrow3} = -2.1 \text{kcal/mol} \) and \( P_{\text{out}} = 0.54 \). For another such mutant, D381A, \( \Delta G_{\text{HIB}} = -2.1 \text{kcal/mol} \) and \( P_{\text{out}} = 0.54 \). For another such mutant, D381A, \( \Delta G_{\text{HIB}} \) does not contribute because alanine cannot act as a hydrogen bond donor, so \( \Delta G_{2\rightarrow3} = \Delta G_{\phi\theta} + \Delta G_{\text{pH}} - \Delta G_{\text{Ala}} = -1.4 \text{kcal/mol} \) and \( P_{\text{out}} = 0.91 \).
the effective 1-step kinetics $k_{\text{obs}} = k_{-2} + k_{\text{on}}L$, where $k_{\text{on}} = k_{+}k_{+}^{-1}(k_{+}^{-1} + k_{-}) = P_{\text{on}}k_{+}^{-2}$. We use this expression to fit the experimental $k_{\text{obs}}$ at low imatinib concentrations (<1.25 μM). However, for the experiments performed over a wider range of imatinib concentrations, we assume only that $k_{+}, k_{+}^{-1}$, and $k_{-2}$ are much greater than $k_{-}$, in which case Eq. S8 simplifies to

$$k_{\text{obs}} = \frac{k_{+}(1 + 10^{\text{pK}_{\text{Im}}})(k_{-}^{-1} + k_{+})}{k_{+}(1 + 10^{\text{pK}_{\text{Im}}})^{-1} + k_{-}^{-1} + k_{-2}} = \frac{k_{+}L}{\frac{1}{k_{+}} + L},$$

where $\zeta = (k_{+}^{-1} + k_{-})k_{-2}$. At each pH value, we independently perform 2-parameter fits of the measured rate constants as a function of imatinib concentration (Fig. 4C, main text). That is, we use Eq. S6 with the pH-dependent $\zeta$ and $k_{+}^{-1}$ as fitting parameters. The nonlinear fit is performed using the Nonlinear-Regression function in Mathematica (7). The fitting yields $k_{+}$ values of 32 s$^{-1}$, 28 s$^{-1}$, 17 s$^{-1}$, 14 s$^{-1}$, and 14 s$^{-1}$ (all with asymptotic sampling error <1.0 s$^{-1}$) for pH 7.0, 7.5, 8.0, 8.5, and 9.0, respectively.

Based on Eq. S6, however, a global 4-parameter ($k_{+}, k_{-}, k_{-2}$, and $\text{pK}_{\text{Im}}$) fit of data at all pH values is unsatisfactory at high pH. This could be explained by the existence of a minority process that has a nonnegligible effect on $k_{\text{obs}}$ at high pH (where $k_{\text{obs}}$ is small):

$$k_{-2} + \text{Abl}(\text{DFG-in}) + \text{imatinib} \rightleftharpoons \text{Abl}(\text{imatinib})^*,$$

where the asterisk indicates that the Abl-imatinib complex is structurally different from that formed by the majority process. This process could correspond to imatinib binding in an alternative mode to the DFG-in conformation, such as is known to occur in Syk kinase (8).

With the assumption that $k_{+2} \gg k_{+3} \gg k_{-3}$, $k_{\text{obs}}$ becomes

$$k_{\text{obs}} = \frac{k_{+}L}{\zeta + L} + \frac{k_{+}L(k_{-}^{-1} + k_{+} + L)}{k_{+} + k_{-}^{-1} + k_{+} + L}.$$  

(S7)

(The meaning of the second term in Eq. S7 is clarified by the observation that when $k_{+}L \ll k_{-}$, this term reduces to $k_{+}Lk_{-}^{-1}$, where $P_{\text{in}} = k_{-}k_{-2}(k_{-}^{-1} + k_{+})$ is the relative population of the DFG-in conformation with respect to the DFG-out. Eq. S7, with global fitting parameters $k_{+}, k_{-}, k_{-2}, k_{+3}$, and $\text{pK}_{\text{Im}}$, fits the data well over the entire pH range (Fig. S8B). The nonlinear fit was performed using the Nonlinear-Regression function in Mathematica, and yielded $k_{+} = 54 \pm 24$ s$^{-1}$, $k_{-} = 36 \pm 5$ s$^{-1}$, $k_{+} = 0.8 \pm 0.2$ μM$^{-1}$s$^{-1}$, $k_{+3} = 0.05 \pm 0.002$ μM$^{-1}$s$^{-1}$, and $\text{pK}_{\text{Im}} = 7.1$; the resultant DFG-flip timescale of 33 ms at pH 7.0 is almost identical to that derived from the independent 2-parameter fit described above.

**Kinetic Scheme: Mathematical Details.** For completeness, in this section we give a more detailed derivation of Eq. S7. With $k_{-3} \ll k_{-2}$ and $k_{-2} \ll k_{-2}L$, we have a kinetic scheme that depends on 4 rate constants ($k_{+}, k_{-}, k_{+2}, k_{+3}$):

$$\text{Abl}(\text{DFG-in}) + \text{imatinib} \rightleftharpoons k_{+}k_{+}^{-1} \text{Abl}(\text{DFG-out}) + \text{imatinib} \rightleftharpoons \text{Abl}(\text{imatinib})^*$$

We assume excess ligand, i.e., that the concentration of imatinib, $L$, is well approximated by a constant. The kinetic scheme is then described by linear differential equations in time $t$, $i$

$$\frac{d}{dt} \begin{pmatrix} C_1 \\ C_2 \\ C_3 \\ C_4 \end{pmatrix} = \begin{pmatrix} A_1 \\ A_2 \\ A_3 \\ A_4 \end{pmatrix},$$

where $C_1, C_2, C_3, C_4$ denote concentrations of \text{Abl}(\text{DFG-in}), \text{Abl}(\text{DFG-out}), \text{Abl}-\text{imatinib} and (\text{Abl}-\text{imatinib})^*$, respectively, and

$$A = \begin{pmatrix} -k_{+}^{-1} - k_{+} + 2L & -k_{+}L & 0 \\ k_{+}^{-1} & -k_{+}L & 0 \\ 0 & 0 & k_{+}L \end{pmatrix}.$$  

The matrix $A$ can be shown to have two nonzero eigenvalues, $\lambda_1$ and $\lambda_2$, given by

$$\lambda_1 = -\frac{b}{2} \left(1 + \sqrt{1 - \frac{4c}{b^2}} \right) \quad \text{and} \quad \lambda_2 = -\frac{b}{2} \left(1 - \sqrt{1 - \frac{4c}{b^2}} \right),$$

where $b = k_{-} + k_{+} + k_{+} + L + k_{+}L$ and $c = L(k_{+} + k_{+} + k_{+}) + k_{+}L)$. The kinetic scheme thus formally leads to biexponential kinetics for Abl-imatinib binding with the two rate constants given by $-\lambda_1$ and $-\lambda_2$.

Our fluorescence experiments are well fit by a single-exponential decay, suggesting the fast rate is too fast to be resolved in the experiment; i.e., we are in the regime where $k_{\text{obs}} = -\lambda_1 \ll -\lambda_2$. In this regime, to a good approximation, $\lambda_2 \approx (1/\lambda_1 + 1/\lambda_2)^{-1} = -\frac{b}{c}$. Thus,

$$k_{\text{obs}} = \lambda_2 \approx \frac{c}{b} \left(\frac{Lk_{+}k_{+}L}{k_{-} + k_{+} + (k_{+} + k_{+})L} + \frac{Lk_{+}(k_{-} + k_{+} + L)}{k_{-} + k_{+} + (k_{+} + k_{+})L} \right).$$

Finally, under the assumption that $k_{+} \gg k_{+}L$ and using the definition $\zeta = (k_{+}^{-1} + k_{+})k_{+}$, this expression for $k_{\text{obs}}$ simplifies directly to that given in Eq. S7.

**Additional Simulation Details.** All simulation systems were set up using GROMACS tools (9, 10), placing the kinase domain in the center of the simulation box and filling the voided space with water molecules. The proteins were oriented with their principal axes running parallel to the edges of the rectangular simulation boxes. The dimensions of the simulation boxes were chosen so that no protein atom was within 6 Å of the edge. Asp-381 was protonated or deprotonated as specified, while protonation states of other residues were set to their default values at pH 7. Na$^+$ and Cl$^-$ ions were added to maintain physiological salinity (150 μM) and to obtain a neutral total charge for the system. All systems were energy minimized using GROMACS 3.2.1 before simulation.

All molecular dynamics simulations were performed by using the parallel MD program Desmond (11) on 64 or 128 dual-processor Opteron nodes connected by a high-speed Infiniband network (Topspin). Desmond employs a particular neutral territory method (12, 13) called the midpoint method (14) to efficiently exploit a high degree of computational parallelism. Long-range electrostatic interactions were computed by the Gaussian split-Ewald method (15) with a Fourier-space solver using a 64 × 64 × 64 mesh of spacing less than 1.5 Å. The Gaussian for charge spreading and force interpolation on the mesh had a standard deviation ($\sigma_0$) of $\approx 1.0$ Å (slightly varying with system size) and was truncated at 3.3$\sigma$. The Gaussian for $k$-space convolution with the charge distribution had a standard deviation between 1.7 and 1.9 Å. The real-space part of the
electrostatic and Lennard–Jones interactions was cut off at 10 Å. The M-SHAKE algorithm was used to constrain the lengths of bonds containing hydrogen. All MD simulations were at constant pressure (1 bar), maintained using a Berendsen barostat, and constant temperature (300 K) maintained using Berendsen thermostats (16). The pressure and temperature control used a relaxation time of 0.5 ps. All simulations used a RESPA integrator (17) with a 2.5-fs time step for the bonded, van der Waals and short-range Coulomb interactions, and a 5-fs time step for the long-range Coulomb interactions. By introducing an orientational potential, the overall orientation of the protein was restrained with its principal axes held approximately parallel to the edges of the simulation box throughout the simulation.

Each simulation is represented by a code number that is given, along with other key simulation details, in Table S1. The human c-Abl kinase domain we simulated consists of residues 230 to 512 inclusive. All simulations based on PDB ID code 2F4J (i.e., simulations 1–7 inclusive) contain the H396P mutation present in that structure. Simulation 8 was based on the kinase domain of PDB ID code 1OPK, and contains the D363N mutation present in that structure. All simulations involving the M290A mutation also contain the W235A mutation. Trp-235 is a solvent-exposed residue located close to the N terminus of the kinase domain of the c-Abl, and asp-381 protonation is common in the DFG-in conformation. The former was estimated based on an active Abl structure (PDB ID code 1OPK) with the catalytic residues removed from the original structure. The pKs of Asp-381 in the DFG-in conformation is estimated to be 6.6, whereas the pKs of Asp-381 in the DFG-out conformation was estimated to be 11.7. This is consistent with the notion that under physiological pH, whereas Asp-381 protonation is common in the DFG-in conformation, it is inherent in the DFG-out conformation.

Additional Details of the Kinetic Measurements of Drug Binding. For the measurement of drug-binding kinetics, we mixed equal volumes of the kinase domain at 100 nM concentration with drug solutions at concentrations ranging from 1 μM to 200 μM for imatinib and 1–25 μM for dasatinib. For each individual binding transient, we mixed a total of 700 μM of reagents in a rapid mixing instrument (Applied Photophysics) with a typical instrumental dead time of ~10 ms. The decrease of protein fluorescence at 350 nm upon excitation at 290 nm was monitored using a HORIBA Jobin Yvon Fluoromax-3 spectrophotometer for 10–20 half-lives of the transient recorded to 1000–2000 data points. The buffer conditions for the experiments were 50 mM Tris (pH range 7.0–9.0) or Bis-Tris (pH range 5.5–6.5), 100 mM NaCl, 1 mM DTT, 5% DMSO. The experiment at high imatinib concentration (Fig. 4C) was carried out with 5% glycerol at 298 K, whereas the experiment at low imatinib concentration (Fig. 4D) was carried out with 15% glycerol at 283 K.

To confirm fast binding events, we also recorded binding transients on a Hi-Tech KinetAsyst stopped-flow instrument with a typical instrumental dead time of ~1–5 ms under identical experimental conditions. The results from both experimental systems were identical.

We recorded at least 3 transients for each experimental condition. Each transient was fitted individually to a single exponential of the form

\[ F(t) = F_0 e^{-k_{off}t} + mt + F_{Eq} \]

Here \( F_0 \) is the amplitude of the change in fluorescence upon drug binding, \( k_{off} \) is the observed rate constant, \( t \) is the time of the reaction, \( m \) is the slope of the baseline, and \( F_{Eq} \) is the fluorescence at equilibrium.

For reactions shorter than 5 sec, the analysis of the residuals of the single-exponential fit did not warrant the inclusion of a sloping baseline in the data fit to account for photobleaching of the tryptophan fluorescence. Observed rate constants were plotted against the final concentration of the drug. For a simple two-state binding process under pseudo first-order conditions, the relationship between the observed rate constant and the ligand concentration is expected to be linear:

\[ k_{obs} = k_{cat}[\text{drug}] + k_{off}. \]

Here \( k_{on} \) is the rate constant for the binding reaction, \( k_{off} \) is the rate constant for the dissociation reaction and [drug] is the drug concentration (19).

**Measurement of Protein–Drug Interaction at Equilibrium.** We measured protein–drug interaction under equilibrium conditions by isothermal titration calorimetry on a MicroCal VP-ITC (MicroCal) essentially as described before (20, 21). Proteins were buffer exchanged into 50 mM Tris, 100 mM NaCl, 0.5 mM TCEP, 15% glycerol, 5% DMSO at pH 5.5–7.5. Imatinib stocks were diluted in the same buffer to 250 μM final concentration and titrated to protein at 10–30 μM concentration at 25 °C. Data were fitted to the single binding site model with the Origin software package (OriginLab). The results are given in Table S2.

**Measurement of the Enzymatic Activity of Src M314A (Corresponding to Abl M290A) Mutant.** Activity of the protein kinases toward the optimal Abi substrate peptide (sequence: EAIYAAPFAKKK) (22) was determined using a continuous spectrophotometric assay, as previously described (23). The Michaelis–Menten constant (\( K_m \)) for the kinase substrate ATP and the maximum catalytic constant \( k_{cat} \) were determined by fitting the observed catalytic rates to the Michaelis–Menten equation (Fig. S2B):

\[ k_{cat}(ATP) = \frac{k_{cat}[ATP]}{K_m + [ATP]} m. \]

We observed that high ATP concentrations decrease kinase activity and therefore we added a sloping baseline—the linear term—to correct for this deviation.

**The Representative Kinase Structure Set.** The representative kinase structure set used in this study was compiled by Nolen et al. (24). We have added structures 2F4J (Abi in an active DFG-in conformation) and 1OPK (c-Abl in an inactive DFG-out conformation) to the original set. The set thus contains 25 active kinase structures and 33 inactive kinase structures. In Fig. S2, the data are from 25 active DFG-in structures (PDB entries 1ATP, 1CKJ, 1CM8, 1DAW, 1HHW, 1HOW, 1IA8, 1IR3, 1JKK, 1JOW, 1K3A, 1KA9, 1M14, 1MRU, 1O6K, 1O9U, 1OLS, 1PKG, 1QMZ, 1VR2, 2ERK, 2F4J, 2PHK, 3LCK), 26 inactive DFG-in structures (1A06, 1AD5, 1B6C, 1ERK, 1F3M, 1FGK, 1FKM, 1H8F, 1HCK, 1JNK, 1JPA, 1K2P, 1KOB, 1M7N, 1MQB, 1MRY, 1MUO, 1NY3, 1O6K, 1OLS, 1OMW, 1PS8, 1QMZ, 1R1W, 1TKI, 1UKH) and 7 inactive DFG-out structures (1FVR, 1IRK, 1LUF, 1OPK, 1RJB, 1T45, 1UWH). Fig. S5 includes only DFG-in structures without ligands bound in or near the ATP-binding site, and comprises 10 active structures (1CKJ, 1HHW, 1H1W, 1H4L, 1IA8, 1IR3, 1IR5, 1JPA, 1K2P, 1KOB, 1M7N, 1MQB, 1MRY, 1MUO, 1NY3, 1O6K, 1OLS, 1OMW, 1PS8, 1QMZ, 1R1W, 1TKI, 1UKH) and 7 inactive DFG-out structures (1FVR, 1IRK, 1LUF, 1OPK, 1RJB, 1T45, 1UWH).
IA8, IJOW, IK9A, 1M14, 1O9U, 1VR2, 2ERK, 3LCK) and 8 inactive structures (1A06, 1F3M, 1FGK, 1M7N, 1P38, 1RIW, 1TKI, 1UKH).

Fig. S1. View into the catalytic cleft of active Abl kinase with Asp-381 protonated (taken from simulation 1a) highlighting the van der Waals interaction between residue Phe-382 and Met-290, which are rendered with van der Waals surfaces. Three conserved residues, Asp-381, Lys-271, and Glu-286 are also shown, with Asp-381 in the ATP-binding site protonated.
Fig. S2. Additional experimental data. (a) The experimentally measured on-rate constants of imatinib binding to c-Abl kinase constructs with and without the SH3-SH2 unit as function of pH. The on rates for c-Abl with regulatory domains are uniformly scaled by a factor of 1.64. The good fit between these 2 datasets indicates that the addition of regulatory domains does not affect the DFG-out population. (b) Comparison of enzyme kinetics of the Src kinase domain of wild-type (red) and M314A mutant (blue). Curves are a variant of Michaelis–Menten fits to the data, which yield $K_m = 0.13$ mM and $k_{cat}^{max} = 581$ min$^{-1}$ for Src wild type (in red), and $K_m = 0.15$ mM and $k_{cat}^{max} = 181$ min$^{-1}$ for Src M314A (in blue).
Fig. S3. Key dihedral angles and interlobe distance as a function of time before, during, and after a DFG flip. The data (from simulation 6b) are sampled at 10-ps intervals and averaged over a running window of 100 ps. All DFG $\phi$ and $\psi$ angles are shown as well as the $\chi_1$ angle of Phe-382. The middle plot is an enlargement of the upper one for times close to the flip, which occurs at 76 ns. Before the DFG flip, other concerted changes of dihedral angles are apparent, at 46 and 57 ns. In the lower plot, the Cα distance of Lys-271 (of the N-lobe) and Ala-424 (of the C-lobe) is plotted as an indicator of the openness of the hinge between the N- and the C-lobes.
Fig. S4. Supporting evidence in resolved kinase structures. (A) A sequence of kinase crystal structures (with PDB ID codes given in parentheses) is shown starting from a DFG-in conformation at the top left and proceeding clockwise to a DFG-out conformation at the bottom left. This sequence resembles the temporal sequence of DFG conformations observed in our simulations (see Fig. 3 in the main text). Explicitly shown in stick representation are the conserved residues Lys-271, Glu-286, and His-361 (c-Abl numbering) as well as the DFG aspartate and phenylalanine (yellow). The sixth conformation shown (DFG-out) is the conformation adopted by imatinib-bound Abl kinase, although imatinib itself is not shown. (B) A simulated DFG flip projected onto Ramachandran plots is shown. The 3 Ramachandran plots together show the $\phi$ and $\psi$ angles for Asp-381, Phe-382, and Gly-383 visited during simulation of the DFG flip (gray dots). The $\phi$–$\psi$ space ranges from $-180^\circ$ to $180^\circ$ in each dimension, with a $60^\circ$ grid spacing. The data are collected every 10 ps from the first 2 ns of simulation 4a, which starts in a DFG-in conformation and after 2 ns reaches a conformation with Phe-382 in the N-pocket, and from the entirety of simulation 6b, which starts from this conformation and ends in a DFG-out conformation via a DFG flip. (A movie of this simulated DFG flip is available as Movie S1.) Arrows indicate the path of conformational change. Also shown are the corresponding $\phi$–$\psi$ angles in the representative X-ray structures: red, orange, and green dots represent structures identified as active DFG-in, inactive DFG-in, and inactive DFG-out conformations, respectively. Two of the largest main-chain changes during the DFG flip are $120^\circ$ in the $\phi$ angles of Asp381 and Gly383; these 2 angles are indicated by the orange arrows in the accompanying illustration of the DFG motif. It is noteworthy that in DFG-in conformations (active or inactive), the aspartate residue is typically located in the less populated left-hand helix region (upper-right part) of the Ramachandran plot, indicative of strained main-chain conformations. This observation is used in the energetic model of DFG conformations described in the main text. (C) pH dependency of the Lys-271-Asp-381 salt bridge (c-Abl numbering) for kinases in DFG-in conformations. The shortest heavy-atom distance between the lysine and the aspartate side chains in a representative set of kinase crystal structures is shown as a function of pH. Only apo crystal structures were included. This figure includes only DFG-in structures without ligands bound in or near the ATP-binding site, and comprises 10 active structures (1CKJ, 1H4L, 1A8B, 1JOW, 1K3A, 1M14, 1O9U, 1VR2, 2ERK, 3LCX) and 8 inactive structures (1A06, 1F3M, 1FGK, 1M7N, 1P3B, 1R1W, 1TK1, 1UKH) from the representative kinase structures set described in the main text.
Fig. S5. Abl-imatinib binding kinetics. (A) Key conformations involved in Abl-imatinib binding. The transition between DFG-in (a) and DFG-out (b) is the DFG flip, which is shown in more detail in Fig. 1. This step is pH dependent as a result of Asp-381, which is solvent exposed in the DFG-in state but not in the DFG-out state. The other step involves the binding of imatinib. In this step, the activation loop undergoes a significant conformational change, such that in the imatinib-bound state (c), Asp-381 is solvent-accessible despite being in a DFG-out conformation. Conformations a, b, and c are based on protein structures 2F4J, 1OPK, and 1IEP, respectively, in the Protein Data Bank. (B) A global fit to the Abl-imatinib binding rates at 5 different pH values. This shows the same experimental data as Fig. 4C in the main text. The observed fluorescence-decay rates for Abl-imatinib binding rates at 5 different pH values are fitted to a global 5-parameter model (the solid lines) described in SI Text.

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Fig. S6. Kinetics of imatinib binding to Abl kinase domain at pH 7.5. (A) Three stopped-flow transients of imatinib binding to Abl kinase domain at 10 °C. The reactions were carried out at final concentrations of 5 μM imatinib and 50 nM Abl in 50 mM Tris (pH 7.5), 100 mM NaCl, 1 mM DTT, 15% glycerol. (B) Residuals of a least-square fit of the data that is shown in A to a single exponential decay with a sloping baseline. The resultant observed rate constant of the reaction $k_{\text{obs}} = 0.241 \text{ s}^{-1}$. The rate constants for the other 2 transients were 0.248 s$^{-1}$ and 0.238 s$^{-1}$. (C) Dependence of observed rate constants on final imatinib concentration at various pH values. The observed rate constants for Abl binding to imatinib at final concentrations between 0.5 μM and 10 μM were fitted to a linear function. The slopes yield the rate constants for the binding reaction ($k_{\text{on}}$) and the intercepts with the y axis yield the rate constants for the dissociation reaction ($k_{\text{off}}$). For example, at pH 7.5, $k_{\text{on}} = 0.048 \text{ s}^{-1} \text{μM}^{-1}$ and $k_{\text{off}} = 0.0086 \text{ s}^{-1}$.
Fig. S7. DFG flips may promote nucleotide binding and release. (a) The principal axis of helix \( \alpha C \) (represented by rods) at 1-ns time intervals from simulation 2a (red, starting from DFG-in, 115-ns trajectory used) and from simulation 8 of equal length starting from the DFG-out crystal structure (PDB ID code 1OPK). The larger helix \( \alpha C \) fluctuations in the DFG-out simulation reflect higher interlobe flexibility, which may facilitate nucleotide binding and release. (b) The crystal structure of DFG-in Abl (red) superimposed onto a conformation in which Phe-382 occupies the N-pocket obtained from our simulations of the DFG flip (green). Neither of these structures contain ADP, but to illustrate the position of its binding site, we have included an ADP molecule (shown in van der Waals representation) in the figure using structural alignment with a kinase–ADP complex structure (PDB ID code 1JBP). The simulation structure has a wide-open ADP-binding site as a result of the large-scale rearrangements, which may facilitate ADP release (or initial ATP binding).
Movie S1. This movie shows a trajectory of a DFG flip starting from the DFG-in and ending at the DFG-out conformation. The DFG aspartate and phenylalanine (Asp-381 and Phe-382) are shown with red and green van der Waals surfaces, respectively. The Abl kinase domain is shown as a ribbon. This trajectory is a concatenation of simulation 4a (first 2 ns) and simulations 6b (first 85 ns), as described in the main text.
Table S1. Summary of simulations

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<th>Simulation</th>
<th>Initial structure (PDB ID code)</th>
<th>Mutations</th>
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<th>Starting DFG conformation</th>
<th>Length, ns</th>
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<td>COOH</td>
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<td>Phe382 does not move into N-pocket</td>
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<td>COO(^-)</td>
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<td>Phe382 moves into N-pocket</td>
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<td>COO(^-)</td>
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<td>DFG-in → out flip at 68 ns and 76 ns, respectively</td>
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</tbody>
</table>

Simulations with the same code number but different letters differ only in their initial thermal velocities, which were assigned randomly. The protonation state of Asp381 is denoted COO\(^-\) (deprotonated) or COOH (protonated). The DFG conformation "N-pocket" denotes a DFG-in conformation in which Phe382 has moved into the N-pocket as discussed in the text. For simulations 3 a–e, the 210-ns figure that appears in the “Length” column represents the aggregate length of all 5 simulations.
Table S2. Thermodynamic properties of imatinib binding to Abl kinase domain at varied pH measured by isothermal titration calorimetry

<table>
<thead>
<tr>
<th>System</th>
<th>N</th>
<th>ΔG (kcal/mol)</th>
<th>ΔH (kcal/mol)</th>
<th>TΔS (kcal/mol)</th>
<th>K_D (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABl (pH 5.5)</td>
<td>0.84</td>
<td>−10.0</td>
<td>−22.6</td>
<td>−12.6</td>
<td>47 ± 8.8</td>
</tr>
<tr>
<td>ABl (pH 6.5)</td>
<td>0.85</td>
<td>−9.6</td>
<td>−15.7</td>
<td>−6.1</td>
<td>88 ± 10.9</td>
</tr>
<tr>
<td>ABl (pH 7.5)</td>
<td>1.01</td>
<td>−10.0</td>
<td>−18.2</td>
<td>−8.2</td>
<td>47 ± 10.2</td>
</tr>
<tr>
<td>ABl (pH 7.5) (2nd measurement)</td>
<td>0.73</td>
<td>−10.4</td>
<td>−17.1</td>
<td>−6.7</td>
<td>23.6 ± 4.7</td>
</tr>
</tbody>
</table>

Here, N refers to the stoichiometry of the binding, ΔG the binding free energy, ΔH and TΔS its enthalpic and entropic contributions, and K_D the equilibrium dissociation constant. We find that the affinity of Abl kinase domain for imatinib is independent of the pH over the pH range studied, which indicates that the relative stability of the drug-bound and drug-free state is unaltered by pH.