Solution Conformations and Dynamics of ABL Kinase-Inhibitor Complexes Determined by NMR Substantiate the Different Binding Modes of Imatinib/Nilotinib and Dasatinib

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Current structural understanding of kinases is largely based on x-ray crystallographic studies, whereas very little data exist on the conformations and dynamics that kinases adopt in the solution state. ABL kinase is an important drug target in the treatment of chronic myelogenous leukemia. Here, we present the first characterization of ABL kinase in complex with three clinical inhibitors (imatinib, nilotinib, and dasatinib) by modern solution NMR techniques. Structural and dynamical results were derived from complete backbone resonance assignments, experimental residual dipolar couplings, and 15N relaxation data. Residual dipolar coupling data on the imatinib and nilotinib complexes show that the activation loop adopts the inactive conformation, whereas the dasatinib complex preserves the active conformation, which does not support contrary predictions based upon molecular modeling. Nanosecond as well as microsecond dynamics can be detected for certain residues in the activation loop in the inactive and active conformation complexes.

Protein kinases play critical roles in intracellular signal transduction pathways, deregulation of which can lead to a variety of pathological states and diseases such as cancer. These enzymes are therefore tightly regulated with multiple layers of control, including phosphorylation, myristoylation, and interaction with SH2 and SH3 or other regulatory domains. Modulation of kinase activity by therapeutic agents is a clinically validated approach for the treatment of chronic myelogenous leukemia. Here, we present the first characterization of ABL kinase in complex with three clinical inhibitors (imatinib, nilotinib, and dasatinib) by modern solution NMR techniques. Structural and dynamical results were derived from complete backbone resonance assignments, experimental residual dipolar couplings, and 15N relaxation data. Residual dipolar coupling data on the imatinib and nilotinib complexes show that the activation loop adopts the inactive conformation, whereas the dasatinib complex preserves the active conformation, which does not support contrary predictions based upon molecular modeling. Nanosecond as well as microsecond dynamics can be detected for certain residues in the activation loop in the inactive and active conformation complexes.

ABL kinase is such a target because the expression of the BCR-ABL fusion protein (caused by unfaithful repair of DNA strand breaks in bone marrow hematopoietic stem cells and subsequent t(9;22) chromosome translocation) leads to life-threatening chronic myelogenous leukemia (1, 2). In BCR-ABL, the breakpoint cluster region BCR protein replaces the N-terminal autoregulatory domain of the Abelson ABL protein to give a constitutively activated tyrosine kinase, which deregulates signal transduction pathways, causing uncontrolled proliferation and impaired differentiation of progenitor cells.

X-ray crystallography has revealed various active and inactive conformational states of kinases, which are implicated in their regulation and modulation by inhibitors (3). The active states are characterized by certain conformations of the activation loop, phosphate-binding loop (P-loop), and helix C, which orient the catalytic machinery to phosphorylate substrates; in the inactive states, one or more of these elements are in different conformations, such that substrate binding and/or catalysis cannot occur. An important determinant is the orientation of the conserved Asp-Phe-Gly motif within the activation loop. For efficient catalysis, this motif adopts a “DFG-in” conformation. In contrast, the “DFG-out” conformation has this motif displaced from the orientation needed for binding the substrate ATP to phosphorylate and activate downstream signaling proteins. Such a DFG-out conformation has been observed in many inactive kinases, including ABL, IRK, KIT, and FLT3 tyrosine kinases (4–7) as well as the serine/threonine kinases p38 MAPK and BRAF (8, 9).

Different kinase inhibitors can bind to and stabilize different kinase conformations, as exemplified in Fig. 1 for different ABL inhibitors. Crystallographic studies have shown that the tyrosine kinase inhibitor imatinib (Glivec®/Gleevec®), a highly effective treatment for chronic phase chronic myelogenous leukemia (10), binds within the catalytic site of the inactive form of ABL with the activation loop in a DFG-out conformation (6, 11–15). This conformation is very similar to that with nilotinib (16) (Tasigna®), a more potent and selective ABL inhibitor developed to inhibit imatinib-resistant mutant forms of BCR-ABL, which frequently emerge in advanced stages of chronic myelogenous leukemia and lead to relapse and disease progression (17). In contrast, crystallographic studies have shown that the multi-targeted ABL and SRC family kinase inhibitor dasatin...
inib (SPRYCEL®) (18) binds to the active DFG-in conformation of ABL (19). However, based on molecular modeling (19, 20), it has been hypothesized that dasatinib can also bind to the inactive DFG-out conformation, and even without experimental support, this notion is becoming established. Crystallographic studies have shown that other promiscuous kinase inhibitors also bind to active conformations of ABL, including compounds of the pyrido[2,3-d]pyrimidine class (21–23) such as PD180970 and the staurosporine derivative AFN941 (11). A clear understanding of the physiologically relevant binding modes of BCR-ABL inhibitor complexes is of utmost importance for the rational design of potent and selective inhibitors that can also counteract the emergence of drug-resistant mutant forms of BCR-ABL (12).

Crystallographic analysis is limited to those biomolecular states that crystallize, which may not capture the full ensemble of conformations that are available in solution under physiologically more relevant conditions. These crystallographic states may be artificially stabilized by crystal contacts while highly dynamical parts of structures remain invisible. In principle, NMR spectroscopy can provide the missing characterization of conformational ensembles and the dynamics of their interconversion. However, NMR work on kinases has been severely limited by their relatively large size, poor solubility, and the fact that they can often be produced only in expression systems that do not allow cost-effective labeling by $^{13}$C, $^{15}$N, and $^2$H isotopes. Only recently have a few NMR studies provided some limited insight on the dynamics of the Eph receptor (24) and p38 MAPK (25) kinases from chemical shift changes and line broadening effects.

In this study, we have applied new techniques such as expression of isotopically labeled ABL kinase in baculovirus-infected insect cells and residual dipolar couplings, which provide precise geometrical information, to characterize the solution conformations and dynamics of the ABL kinase domain in complex with the three clinically used inhibitors: imatinib, nilotinib, and dasatinib.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—Expression and purification of uniform and amino acid-selective $^{13}$C/$^{15}$N isotope-labeled ABL kinase in baculovirus-infected insect cells were carried out as described previously using the construct His$_{6}$-TEVsite-GAMDP-hABL(Ser$^{259}$-Ser$^{269}$) (26, 27).
Solution Conformations of ABL in Complex with Inhibitors

Selectively \([U^{13}C/15N]Phe\)-Gly-Met-Tyr (FGMY) and \([^{13}CO]Leu\)-Thr-labeled ABL kinase was expressed in a custom-made BioExpress 2000 medium containing \([U^{13}C/15N]Phe\)-Gly-Met-Tyr and \([^{13}CO]Leu\)-Thr (Cambridge Isotope Laboratories, Inc.) as described (28), but without addition of imatinib to the culture medium. FGMY-labeled His-ABL kinase was isolated by nickel affinity chromatography (nickel-nitrilotriacetic acid, Qiagen) with imidazole elution, yielding 20 mg of heterogeneously phosphorylated kinase from two 0.5-liter cultures. Incubation with ActEVTM protease (100 units/mg of His-ABL; Invitrogen) and YOP protein-tyrosine phosphatase (1000 units/ml of reaction; New England Biolabs) for 15 h at 6 °C removed the His tag and dephosphorylated the protein. Inhibitor (imatinib, nilotinib, dasatinib, PD180970, or AFN941) was added to aliquots of the reaction from 20 mm stock solutions in Me2SO. The ABL complexes were then purified by size exclusion chromatography (Superdex 75 HR10/30 column, GE Healthcare) in 20 mm BisTris, 150 mm NaCl, 1 mm EDTA, and 3 mm TCEP (pH 6.5), except for the ABL-PD180970 complex, for which 20 mm Tris, 100 mm NaCl, 1 mm EDTA, and 2.5 mm TCEP (pH 7.6) was used. Purified complexes were concentrated (Ultrafree-0.5, 10 kDa, Millipore) to 230–330 μM. Protein concentration, purity, and stoichiometry were determined by high pressure liquid chromatography for each complex. Liquid chromatography/mass spectrometry analysis showed an incorporation of \(^{13}C/^{15}N\) label of 95% and 12% residual monophosphorylation for the purified FGMY-labeled ABL kinase.

**NMR Samples**—Uniformly \(^{13}C/^{15}N\)- and \(^{15}N\)-labeled samples of ABL-imatinib complexes (1:1) were prepared as 0.4 mm solutions in 250 μl of 95% H2O and 5% D2O, 20 mm BisTris, 100 mm NaCl, 2 mm EDTA, and 3 mm dithiothreitol or TCEP (pH 6.5). Selectively labeled samples of imatinib, nilotinib, dasatinib, and PD180970 complexes (1:1) were prepared as solutions (0.32, 0.32, 0.22, and 0.22 mm respectively) in either 95% H2O and 5% D2O, 20 mm BisTris, 150 mm NaCl, 2 mm EDTA, and 3 mm dithiothreitol or TCEP (pH 6.5) (imatinib, nilotinib, and dasatinib) or 95% H2O and 5% D2O, 20 mm Tris, 100 mm NaCl, 1 mm EDTA, and 2.5 mm TCEP (pH 7.6) (PD180970). Similar preparations were tested for an ABL-AFN941 complex. However, this complex precipitated even at the low concentration of 0.1 mM, and no NMR data could be acquired. Non-isotopic samples of selectively labeled imatinib and nilotinib (dasatinib) complexes were prepared by adding 30 mg/ml (20 mg/ml) filamentous phage Pf1 (Asla Biotech).

**NMR Resonance Assignments and Measurement of Residual Dipolar Couplings (RDC) Values**—NMR spectra were recorded at 293 K on Bruker DRX 600 MHz (with and without a Cryo-Probe) and 800 MHz (equipped with a TCI CryoProbe) spectrometers. All spectrometers were equipped with triple-resonance, triple-axis pulsed-field gradient probes. Backbone assignments followed standard triple-resonance strategies with two- and three-dimensional experiments, including HNCO, HNCA, HN(CO)CA, and \(^{15}N\)-edited \(^{1}H\)-\(^{1}H\) nuclear Overhauser effect (NOE) spectroscopy. All NMR data were processed using the NMRPipe suite of programs (29) and analyzed with NMRView (30) to obtain assignments. RDCs were obtained as differences in the splitting observed in the two-dimensional \(^{1}H\)-\(^{15}N\) in-phase anti-phase experiments (31) under anisotropic and isotropic conditions.

**NMR Relaxation Experiments and Analysis**—Standard \(^{15}N\) relaxation measurements (T1/T2, \(^{1}H\)-\(^{15}N\) NOE) were recorded on the ABL-imatinib complex (uniformly and selectively labeled samples) at 800 MHz. T1/T2 decay curves were fitted by an in-house written routine implemented in MATLAB (MathWorks, Inc.) using a simple search minimization and Monte Carlo estimation of errors (see Fig. 7, A and B). Lipari-Szabo model-free analysis of \(^{15}N\) relaxation data was achieved using the TENSOR2 suite of programs (supplemental Fig. 1) (32).

**RESULTS**

**Resonance Assignment of the ABL-Imatinib Complex**—Assignment of backbone NMR resonances was initially performed for the ABL kinase domain (GAMDP-Ser229–Ser500, human ABL1, isoform 1A, 32 kDa) in its non-phosphorylated form and in complex with imatinib. We have shown previously that efficient production of well folded ABL with uniform \(^{13}C/^{15}N\) isotope labeling is possible by the baculovirus Sf9 insect cell expression system (26). NMR analysis of the ABL complex was difficult for two reasons. 1) Because of solubility problems, ABL concentrations in the NMR samples had to be less than ~0.4 mm. 2) The assignment had to be carried out using protonated protein because cost-effective deuterium labeling is currently not possible in the insect cell system. Consequently, the short transverse relaxation times of the 32-kDa complex allowed only HNCO, HNCA, HNCOCA, and \(^{15}N\)-edited NOE spectroscopy backbone assignment experiments and prevented the use of CBCA-type experiments, which would have yielded distinctive amino acid-type information (33). Supplemental information about amino acid types was therefore obtained from a total of 15 additional, selectively labeled ABL samples. Further details on these samples and the obtained chemical shifts are described elsewhere (34). The available assignments comprise 96% of all backbone \(^{1}H\), \(^{13}N\), \(^{13}C\), and \(^{13}CO\) resonances, covering 254 of the 264 non-proline residues (Fig. 2). Unassigned residues consist of the N-terminal glycine, several residues within the activation loop, and His361 lining the imatinib-binding surface. Line broadening of adjacent residues indicates that most of the missing residues are broadened beyond detection because of intermediate conformational exchange.

**Design of Isotope Labeling Scheme for the Study of Various Inhibitor Complexes**—After fully assigning the resonances of the ABL-imatinib complex, a strategy employing selective amino acid labeling was devised for the rapid and unambiguous resonance assignment of key residues in the other inhibitor complexes. To select the best suited labeling scheme, residual \(^{1}H\)-\(^{15}N\) dipolar couplings were predicted based upon the crystal structures of the various inhibitor complexes and the orientation tensor of the ABL-imatinib complex measured in Pf1 phages (35). Residues for selective labeling were then chosen to maximize the differences of the predicted dipolar couplings in the “inactive” DFG-out and “active” DFG-in conformations. Thus, maximal experimental differentiation by RDC data between these two conformations should be achieved.
The chosen labeling scheme (FGMY) consists of uniform $^{13}$C/$^{15}$N labeling for Phe, Gly, Met, and Tyr and specific $^{13}$CO labeling for Thr and Leu residues. FGMY labeling covers five residues (Gly$^{249}$, Gly$^{250}$, Gly$^{251}$, Tyr$^{253}$, and Gly$^{254}$) in the P-loop and seven key residues (Phe$^{382}$, Gly$^{383}$, Ser$^{385}$, Met$^{388}$, and Tyr$^{393}$) are preceded by $^{13}$CO-labeled Leu or Thr at a sample concentration of 0.4 mM. The ultimate assignment of resonances was achieved by a combination of three- and two-dimensional versions of HNCA, HNCO, and HN(CO)CA experiments.

**Chemical Shift Analysis**—The chemical shift of a nucleus is a sensitive probe of its local environment and can therefore serve as a fingerprint for different molecular conformations. Comparison of the HSQC fingerprint spectra of the ABL-inhibitor complexes demonstrates that the two DFG-out complexes (imatinib and nilotinib) possess a high degree of similarity (Fig. 3B), but are very distinct from the dasatinib DFG-in complex (Fig. 3A). The HSQC spectrum of the dasatinib complex has a P-loop (red), and helix C (gold) is shown at the top. Boldface helices indicate $\alpha$-helices, whereas dotted helices indicate $3_{10}$-helices. Unassigned residues are underlined. The spectrum was acquired for 1 h at a sample concentration of $\sim 0.4$ mM.

The amino acid sequence of the ABL kinase domain (GAMDP-GLGQ) with its secondary structure and the mentioned activation loop (magenta), P-loop (red), and helix C (gold) is shown at the top. The spectrum was acquired for 1 h at a sample concentration of $\sim 0.4$ mM.
FIGURE 3. Chemical shift analysis of selectively labeled (FGMY) ABL-inhibitor complexes. A and B, extracted region from the $^1$H-15N HSQC spectra of selectively FGMY-labeled ABL kinase in complex with imatinib (A; black) and dasatinib (B; black). Resonances of the ABL-nilotinib complex (green) are shown for comparison. Residues labeled in red show the largest chemical shift changes. Asterisks indicate unassigned resonances of a low molecular mass impurity. C, weighted chemical shift differences $\Delta\delta_{\text{ave}} = (\Delta\delta_{\text{N}}^2(N)/50 + \Delta\delta_{\text{H}}^2(H)/2)^{1/2}$ between imatinib and nilotinib (inactive-inactive; black), imatinib and dasatinib (inactive-active; red), and dasatinib and PD180970 (active-active; blue) complexes. Open circles for Gly383/Gly398 indicate ambiguous assignments for dasatinib. Boxes at the top indicate secondary structure elements showing $\beta$-sheets (black) and helices (white). The spectra were acquired for 6 h at a sample concentration of $\sim 0.3 \text{mM}$ ($\sim 0.2 \text{mM}$) for imatinib and nilotinib (dasatinib) complexes.
Between imatinib and nilotinib, chemical shift differences larger than 0.1 ppm are detected only for Met290, Phe317, and Gly383, which are in direct contact with the inhibitors. Much stronger differences are observed between the dasatinib and imatinib complexes in the region of the P-loop around Gly250, the hinge region residues Phe317 and Gly321, and the activation loop around Met388. Whereas the residues in the P-loop and hinge region participate in direct interactions with the inhibitor, the affected residues in the activation loop (Met388 and Gly390) are not in direct contact, and hence, their chemical shift changes are in agreement with an allosteric reorientation of the activation loop (Fig. 4).

Residual Dipolar Couplings and Solution Structures—A more quantitative description of the conformations in the different inhibitor complexes was obtained by RDCs. These can be induced in solution by the weak alignment of biomacromolecules (36) and provide a measure of the orientation of internuclear vectors with respect to a fixed coordinate system. Thus, the RDC of the amide N-H bond ($J^N_{\text{NH}}$) is given as follows:

$$J^N_{\text{NH}} = J^N_{\text{NH},\text{max}}(P_2(\cos \theta) + \eta/2 \sin^2 \theta \cos 2\phi),$$

where $J^N_{\text{NH},\text{max}}$ is a constant depending on the degree of orientation, $P_2$ is the second Legendre polynomial, $\eta$ is the rhombicity of the alignment tensor, and $\theta$ and $\phi$ are polar coordinates of the N-H vector in the principal axis system of the alignment tensor (36).

Because RDCs can be measured with high precision, their geometric dependence makes them a powerful tool to study solution conformations and compare them with other structural models such as solid-state X-ray crystal structures.

Weak alignment of the selectively labeled ABL-inhibitor complexes was achieved by the addition of filamentous bacteriophage Pf1 (35). Large $J^N_{\text{NH}}$ RDCs (~30 Hz) were obtained for the imatinib, nilotinib, and dasatinib complexes, which indicated substantial alignment and allowed for high sensitivity detection. For the PD180970 complex, the spectral quality was insufficient, and several key resonances were unobservable because of intermediate conformational exchange.

For the imatinib and nilotinib complexes (DFG-out), the RDC values are strikingly similar throughout the protein (Fig. 5A), implying very similar solution structures and dynamics for the hinge region residues Phe317 and Gly321, and the activation loop around Met388. Whereas the residues in the P-loop and hinge region participate in direct interactions with the inhibitor, the affected residues in the activation loop (Met388 and Gly390) are not in direct contact, and hence, their chemical shift changes are in agreement with an allosteric reorientation of the activation loop (Fig. 4).

**FIGURE 4.** Mapping of largely shifted residues on three-dimensional structures of ABL-inhibitor complexes. Overlaid structures of ABL-inhibitor complexes are shown. A, ABL-imatinib (gray; Protein Data Bank code 1IEP) and ABL-nilotinib (yellow; code 3CS9). The P-loop, activation loop, and inhibitors are colored in blue and green for imatinib and nilotinib complexes, respectively. B, ABL-imatinib (gray; code 1IEP) and ABL-dasatinib (green; code 2GQG, molecule B). The P-loop, activation loop, and inhibitors are colored in blue and yellow for imatinib and dasatinib complexes, respectively. Residues indicated in red show relatively larger changes in the chemical shifts (>0.1 ppm for A and >0.3 for B).

**FIGURE 5.** Analysis of RDCs (measured in Pf1 phages) of selectively labeled (FGMY) ABL-inhibitor complexes. Shown is a comparison of the experimental $J^N_{\text{HN}}$ RDCs obtained for imatinib and nilotinib complexes (A) and imatinib and dasatinib complexes (B) shown along the primary sequence. Open circles for Gly383/Gly384 indicate ambiguous assignments for dasatinib. Error bars at the top indicate secondary structure elements: $\beta$-sheets (black) and helices (white).
both complexes. In marked contrast, RDCs for the dasatinib complex (Fig. 5B) differ substantially from those for the imatinib and nilotinib complexes in both the activation loop and the P-loop. Thus, the solution conformation of the dasatinib complex clearly differs from that of the imatinib and nilotinib complexes, which corroborates the results of the chemical shift analysis.

To characterize the conformations of the activation loop (Asp382-Pro401), P-loop (Lys247-Glu259), and hinge region (Phe317-Leu323) in detail, theoretical RDC values were calculated for each complex. For this, alignment tensors were determined employing a linear fit procedure (37) using the respective crystal structures and the measured RDCs, but excluding the activation loop, P-loop, hinge region, and the flexible residues at the N terminus (<Met237) and C terminus (>Phe193). Using these alignment tensors together with the crystal coordinates, RDC values were predicted for the entire protein, with the previously excluded regions included. These theoretical values were then compared with the experimental RDC values for the imatinib, nilotinib, and dasatinib complexes (Fig. 6).

For the imatinib and nilotinib complexes (Fig. 6, A and B), it is evident that, besides the flexible N-terminal region, all RDC values throughout the entire protein including the loop regions are in perfect agreement with the crystal structures. In particular, this is the case for Phe382, Gly383, Ser385, Met388, Gly390, Tyr393, and Gly398 in the activation loop. This demonstrates that the ABL-imatinib and ABL-nilotinib complexes adopt the inactive DFG-out conformation in solution and that any dynamic variations from the crystal structure coordinates must be small.

Taking into account the slightly larger experimental errors for the dasatinib complex, the agreement between measured RDC values and those predicted from the crystal structure (Protein Data Bank code 2GQG) is also very good for this complex. The asymmetric unit of the crystal structure 2GQG contains two ABL molecules, one in the phosphorylated form (molecule A; phospho-Tyr393) and one in the non-phosphorylated form (molecule B). Both structures have almost identical backbone conformations. Predictions are shown in Fig. 6C for the non-phosphorylated form because the ABL protein used for solution NMR was also non-phosphorylated. Besides the flexible N and C termini, moderate deviations between measured and predicted RDCs outside of the experimental error are observed only for turn residues Gly442, Gly444, and Gly463, which are all part of loop regions. For all other unambiguous assignments, very close agreement is observed. In particular, Phe382, Met388, and Gly390 within the activation loop could be detected and assigned unambiguously. Because of exchange broadening (see below), a further glycine resonance could only be assigned in an ambiguous way either to Gly383 or Gly398 (shown as open circles in Fig. 6, C–F). However, for both the unambiguous and the two possible ambiguous assignments in the activation loop, the experimental RDCs correspond very closely to the prediction of the 2GQG structure. Very similar agreement is found when the experimental data are compared with the crystal conformation of the dasatinib complex with phosphorylated ABL (2GQG, molecule A) (data not shown). Thus, we conclude that, in solution, the activation loop of the dasatinib complex predominates in the active DFG-in conformation corresponding to that of the 2GQG crystal structure.

To estimate the discriminative power of RDC values for different conformations of the activation loop, we compared the experimental RDCs of the dasatinib complex with those predicted from both the “inactive state” protein in complex with imatinib (Fig. 6D) and the “active state” protein in complex with structurally unrelated ABL kinase inhibitors PD180970 (Fig. 6E) and AFN941 (Fig. 6F). For the inactive state imatinib complex, all of the predictions for the activation loop region are outside of the error limits of the measured RDCs, showing that the dasatinib complex does not sample the inactive imatinib conformation to a significant extent.
Solution Conformations of ABL in Complex with Inhibitors

In the fast chemical exchange regime, the RDC values of an averaging ensemble of structures are given by the average over the RDCs of the different conformations. Using an error estimate of 3 Hz for both experimental and predicted data, we calculate from the large differences and, in some cases, the different sign between measured and predicted data (e.g. for Phe382 and Gly383) that we would detect an inactive imatinib conformation if it were populated by more than ~15%. However, our experimental data are fully compatible with the ABLDasatinib complex being exclusively in the active conformation.

The crystal structures of the PD180970 and AFN941 complexes show that these inhibitors also bind to ABL in the active conformation, although the path of the activation loop in these complexes is more variable than in the dasatinib complex. Thus, the PD180970 complex is in the active conformation with respect to most of the activation loop, but the crystal structure for this complex shows the DFG motif in a conformation in which the Asp side chain is flipped over to form a hydrogen bond with a main chain carbonyl group. This conformation does not support the binding of ATP, yet is completely different from the DFG-out conformation (11). The experimental RDC values for the dasatinib complex strongly deviate in the DFG region (positions 381–383) from the predicted RDC values (Fig. 6E). Therefore, we conclude that the PD180970 crystal DFG conformation is not highly populated in solution by the dasatinib complex. In contrast, the predictions from the ABLAFN941 complex (Fig. 6D), which has a typical active DFG-in conformation similar to 2GQG, are closer to those from the dasatinib structure and the experimental RDCs in the activated conformation similar to 2GQG, are closer to those from the crystal structure shows the P-loop region, probably because the crystal structure shows disorder in this region and only part of the P-loop could be seen in the electron density.

In summary, all RDC data indicate that for the imatinib and nilotinib complexes, the ensembles of solution conformations are very close to the static structures observed in the crystal. The RDC data also unambiguously show that the conformational ensemble of the dasatinib complex in solution clusters around the active DFG-in conformation observed in the crystal and that inactive DFG-out conformations are not sampled to a significant extent.

**Backbone Dynamics**—The backbone of the ABL-imatinib complex was characterized by 15N relaxation experiments (Fig. 7). Decreases in 15N T1 and [1H]-15N NOE values and an increase in T2 values at the N terminus before Met237 and at the C terminus after Phe493 indicate high nanosecond mobility at both termini. A high rigidity throughout

![Figure 7. 15N relaxation data of the ABL-imatinib complex.](Image)

The backbone relaxation data was carried out by the program TENSOR2 (32), yielding the isotropic rotational correlation time (τᵣ), the Lipari-Szabo (38), subnanosecond order parameters (S²), and exchange contributions to transverse relaxation from conformational exchange on the micro- to millisecond range (supplemental Fig. 1). The resulting value for τᵣ of 21 ns is slightly larger than expected for a molecule of the size of ABL tumbling in aqueous solution at 20 °C. Presumably, this larger value is caused by the onset of aggregation, which occurs at the concentration of 0.4 mM used for the experiments. However, further dilution was not attempted because of the reduced sensitivity that would result.
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The higher mobility in the region of the activation loop is reflected in the TENSOR2 analysis both by a reduced order parameter ($S^2$) of $-0.6$–$0.7$ and contributions from chemical exchange broadening on the microsecond time scale of up to $25$–$30$ Hz.

Such chemical exchange effects are clearly visible as a broadening and weakening of the resonance lines in the $^1$H-$^15$N HSQC spectra. Fig. 8 shows the respective peaks of the activation loop and P-loop for the imatinib, nilotinib, and dasatinib complexes. For imatinib, prominent line broadening is observed for Met$^{388}$ and Tyr$^{393}$, which cannot be attributed to hydrogen exchange with water because exchange peaks with water are absent in the $^{15}$N-edited NOE spectra. Hence, the line broadening is caused by conformational exchange on the microsecond time scale of chemical shifts. The broadening of Tyr$^{393}$ is particularly interesting because this residue becomes phosphorylated in the activated complex. Similar line broadening is observed for the nilotinib complex. However, much more pronounced broadening occurs for the dasatinib complex, e.g. Ser$^{385}$, and Tyr$^{393}$ could not be detected at all. This clearly indicates a differing dynamic behavior of the activation loop in the dasatinib complex.

Within the P-loop, weak exchange broadening is observed for Gly$^{249}$ and Tyr$^{253}$ in the case of imatinib and nilotinib (Fig. 8). Again, much stronger exchange broadening occurs for Gly$^{249}$ in the dasatinib complex. Thus, also the P-loop appears more mobile in the case of the active state inhibitor dasatinib.

These relaxation results on the ABL-inhibitor complexes are significant because they directly show the presence of dynamic processes in several regions of the protein, including the activation loop. It should be noted, however, that although the effects of line broadening are considerable, they do not necessarily imply that the populations of the other conformations, which are exchanging with main species, are large. Admixtures of populations on the order of $1\%$ can lead to significant broadening effects (39). Thus, the detection of such motions by line broadening is not contradictory to the finding from the RDC analysis that the major part of the solution ensemble is close to the crystal structure.

DISCUSSION

Our results compose the first detailed structural characterization of protein kinase-inhibitor complexes in solution. They are based on the availability of the almost complete assignment of the backbone resonances of the ABL-imatinib complex and partial resonance assignments of the ABL-nilotinib, ABL-dasatinib, and ABL-PD180970 complexes. This allowed an analysis of chemical shift perturbations, RDC, and $^{15}$N relaxation data. For the imatinib and nilotinib complexes, the ensemble of solution conformations closely resembles the static inactive DFG-out structure determined in the crystal, although residual mobility of the activation loop can be detected from $^{15}$N relaxation data and the line broadening of some resonances.

For the dasatinib complex, the RDC data clearly show that the ensemble of solution conformations is close to the active DFG-in structure. However, line broadening effects around Ser$^{385}$ and Tyr$^{393}$ in the activation loop and Gly$^{249}$ in the P-loop indicate the presence of microsecond to millisecond motions in these regions. Relaxation dispersion can reveal the time scale and chemical shift differences of the species involved in the exchange (39). Because of the low solubility ($\approx0.2$ mM) of the dasatinib complex and the weak intensity of the signals, such experiments were not attempted. Nevertheless, based on the close agreement between measured RDCs and the prediction according to the active state conformation, the amplitude of these microsecond to millisecond motions and/or the populations of the exchanging minor conformations should be small. Such minor conformations may be the result of small rearrangements of the backbone or the side chains or variations in hydrogen bond geometries.

Based on molecular modeling and molecular dynamics studies, it has been hypothesized that dasatinib can bind to both the active DFG-in and inactive DFG-out conformations of ABL (19, 20). This notion has become widespread despite the absence of supportive experimental evidence and the fact that the x-ray structure of the ABL-dasatinib complex shows only the active ABL conformation. This study is the first to actually assess the extent of DFG-out conformation in the ABL-dasatinib complex in solution. No significant admixture of the DFG-out conformation is detectable from the measured RDC values. In a further experiment (data not shown), we displaced imatinib by adding dasatinib in high excess to the ABL-imatinib complex rather than adding dasatinib directly to unliganded ABL. Even when offering the preformed inactive DFG-out state to dasatinib in this manner, the resulting ensemble of conformations is indistinguishable from the ensemble observed when adding dasatinib to unliganded ABL.

This study was performed with non-phosphorylated protein, which was necessary to allow the protein to also adopt the inactive DFG-out conformation. The phosphorylation of Tyr$^{393}$ in the activation loop stabilizes the active conformation of the protein by forming interactions with neighboring side chains (40). It can be expected that this will reduce the flexibility of the dasatinib complex, thereby narrowing the ensemble of the
active DFG-in conformations even further. For the same reason, any propensity of the ABL-dasatinib complex to adopt the inactive DFG-out conformation should be even more reduced.

It is generally believed that there is only one active state, which satisfies the requirement to have all essential elements correctly orientated for efficient catalysis. In contrast, multiple inactive states may exist. This is supported by experimental findings for both ABL and SRC kinases in complex with various ligands (41). Our findings of very similar inactive states for the imatinib and nilotinib complexes do not contradict this notion; although multiple ligand-dependent inactive states may exist, we have shown that the inactive state with a particular ligand is well defined and resembles closely the conformation observed in the crystal. However, these inactive conformations are not completely rigid because we still observed high nanosecond backbone flexibility within the activation loop despite the fact that the RDC values indicate that the ensemble average is close to the x-ray structure.

The observed flexibility seen in both the active and inactive states is likely to be an intrinsic requirement for catalytic activity and for the transition between the active and inactive conformations of ABL as well as other kinases (42). Indeed, molecular dynamics calculations have shown that the various inactive ABL kinase conformations may be necessary intermediates in this transition (41).

Considering the tendency of different kinases to adopt different inactive states, e.g. either DFG-in or DFG-out conformations, the free energy differences between these states appear to vary significantly between kinases. For example, whereas DFG-out conformations seem extraordinarily stable for certain ABL complexes, such conformations, although possible, have a high thermodynamic penalty in SRC complexes (43). The reason for this strikingly differing behavior is unknown and cannot be attributed to a few individual differing amino acids. The role of such residues in the neighborhood of the DFG motif or elsewhere and the overall energetics of the different states cannot be determined from the crystal structures alone. NMR-derived dynamic information, as obtained here, should lead us to better understand these systems and to comprehend why certain inactive conformations are more or less favorable in some kinases relative to others (21).

To understand how point mutations cause patient resistance to imatinib and eventual relapse is also of crucial importance because the efficacy of inhibitors may be related to the free energy landscape of the various inactive states (43). A comprehensive description of this behavior is a fundamental prerequisite for a more rational design of potent new drugs.

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