

Dynamics in the p38 α MAP Kinase–SB203580 Complex Observed by Liquid-State NMR Spectroscopy**

Valerie S. Honndorf, Nicolas Coudeville, Stefan Laufer, Stefan Becker, and Christian Griesinger*

Diaryl-heterocycle compounds were the first small-molecule inhibitors targeting mitogen-activated protein (MAP) kinases.^[1] The diaryl heterocycle SB203580 binds in an adenosine triphosphate (ATP) competitive manner to inactive and active p38 α MAP kinase with similar IC₅₀ values.^[2] A dissociation constant (K_D) of 11.5 nM was reported for the inactive form.^[3] Crystallographic studies showed that SB203580 binds in the ATP-binding site of p38 α . Similarly to ATP, the pyridine nitrogen atom of SB203580 forms a hydrogen bond to the backbone amide of Met109 from the hinge region.^[4] In contrast to the binding of ATP, the positively charged side chain NH₃⁺ group of Lys53 forms a hydrogen bond to the imidazole nitrogen atom of SB203580, the fluorophenyl group is situated in the hydrophobic pocket I, and the methylsulfinyl group contacts the phosphate binding region below the glycine-rich loop while the linked phenyl ring forms stacking interactions with Tyr35 in the glycine-rich loop (Figure 1).^[4,5]

In the crystal structure (PDB code: 1A9U), the interacting residues are fully occupied with rather low B factors. This clearly suggests the formation of a very rigid complex between the inhibitor and the protein. Similar observations are made for the crystal structures of several other small-molecule inhibitor complexes of p38 α as well as of the free form of the protein.^[5,6] The hinge region seems to be the most important anchor point for diaryl-heterocycle kinase inhibitors and its sequence is very specific for each MAP kinase.^[7] To obtain a deeper insight into the effect of the conserved pharmacophore of these inhibitors on the dynamics of their interaction with inactive p38 α in solution, we performed NMR measurements on the prototypical SB203580/p38 α MAP kinase complex.^[8]

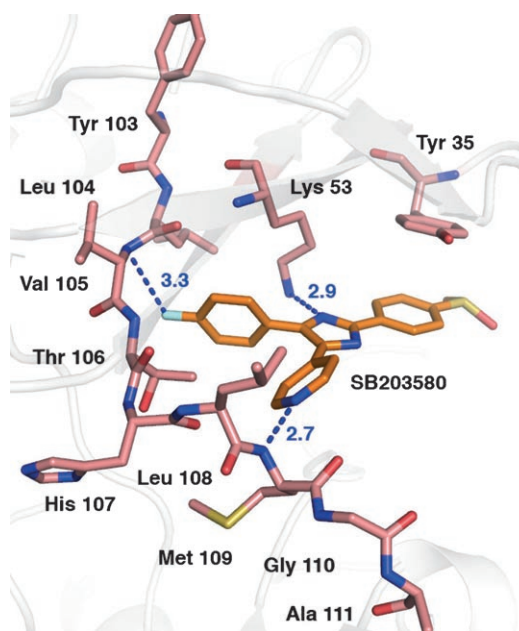


Figure 1. Structure of p38 α in a complex with SB203580 (Protein Data Bank (PDB) access code: 1A9U). SB203580, as well as residues involved in the interaction, are represented as sticks; hydrogen bonds are represented as blue dashed lines.

To verify the assignment of the free form of p38 α ^[9] and to assign the p38 α /SB203580 complex, we recorded a TROSY-HNCA spectrum for triple-labeled samples. As reported before,^[10] only 75 % of the expected resonances were observable, probably because of intrinsic dynamic heterogeneity. About 70 % of the observable resonances were assigned for both forms. Comparison of the ¹H–¹⁵N TROSY spectra of p38 α in its free and inhibitor-bound forms revealed chemical-shift perturbation for a limited set of residues, all located near the binding site of SB203580 in the crystal structure (Figure 2), thereby confirming previous results.^[10]

To detect potential conformational changes induced by SB203580 bound to p38 α in solution, we measured one-bond ¹D_{1H–15N} residual dipolar couplings (RDCs) for p38 α in the free and inhibitor-bound forms in a weakly oriented medium (with 20 mg mL^{−1} bacteriophage Pf1). The large size of p38 α means that the measurement of RDCs leads to large errors. Only RDC values with an error lower than 5 Hz^[11] were taken into account; this resulted in an ensemble of 73 couplings for the free form and 43 couplings for the inhibitor-bound form, which exhibits more dynamic heterogeneity. Each set of couplings was used to determine the alignment tensor by using the singular-value decomposition approach with the

[*] Dipl.-Chem. V. S. Honndorf, Dr. N. Coudeville, Dr. S. Becker, Prof. Dr. C. Griesinger
Department of NMR-based Structural Biology
Max-Planck-Institute for Biophysical Chemistry
Am Fassberg 11, 37077 Göttingen (Germany)
Fax: (+49) 551-201-2202
E-mail: cigr@nmr.mpibpc.mpg.de

Prof. Dr. S. Laufer
Department of Pharmaceutical and Medicinal Chemistry
Institute of Pharmacy, Eberhard-Karls-University
Auf der Morgenstelle 8, 72076 Tübingen (Germany)

[**] MAP: mitogen-activated protein. We thank Prof. Dr. Thilo Stehle, Eberhard-Karls-University, Tübingen, for the clone of p38 α MAPK. This work was supported by the Max Planck Society, the Deutsche Forschungsgemeinschaft (grant no.: GRK1034), and the Fonds der Chemischen Industrie.

Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.

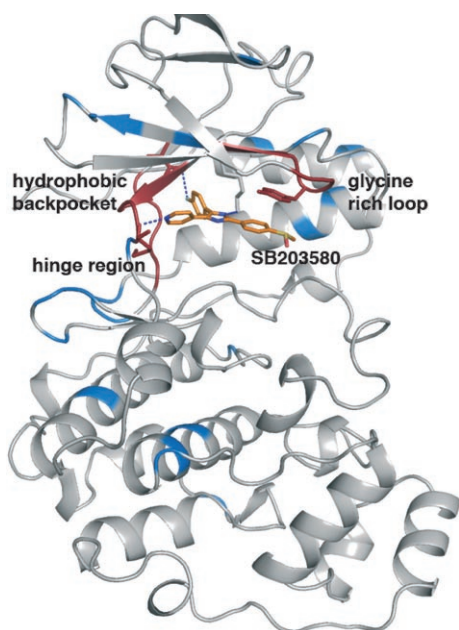


Figure 2. Mapping on the crystal structure of p38 α /SB203580 (PDB code: 1A9U) of residues (in blue) significantly affected upon inhibitor binding to p38 α in solution. Residues marked in red have resonances that disappear after inhibitor binding.

PALES software.^[12] Both sets were fitted to the crystal structure of free p38 α (PDB code: 1P38).^[13] In both cases, the RDC ensemble fits rather well to the crystal structure with correlation coefficients of 0.94 and 0.92 ($Q=0.25$ and $Q=0.31$) for the free and inhibitor-bound forms, respectively (see Table I in the Supporting Information). These values indicate that the average overall conformations of p38 α in solution, in the free and inhibitor-bound forms, are very similar to the conformation observed in the crystal (Figure 3).

However, for the free structure, the dipolar couplings of Gly110 and Ala111, which were determined to be -5.9 and 19.6 Hz, respectively, had to be excluded to obtain the excellent fit reported above. Removal of these two dipolar couplings changed the Q value from 0.57 to 0.25, a result indicating that the solution structure for this specific region of the protein differs significantly from the crystal structure. Indeed, the Gly110 and Ala111 residues would exhibit RDC values of 21.6 and -16.9 Hz, respectively, if they had the local conformation predicted in the 1P38 crystal structure. It has been reported that, upon binding of particular inhibitors (containing hydrogen-bond acceptors addressing the hinge region), a peptide flip is induced in which the φ, ψ angles of Met109 and Gly110 are changed.^[14] Fitting of our RDC data with one of the crystal structures exhibiting the peptide flip (PDB code: 1OVE) led to a surprisingly good correlation of 0.83. Interestingly, the RDC values predicted from the 1OVE crystal structure for Gly110 and Ala111 (-7.6 and 22.9 Hz, respectively) are very close to the experimental ones (-5.9 and 19.6 Hz, respectively). This observation indicates that, in the free solution structure of p38 α , both residues predominantly adopt a conformation that differs from the crystal structure of the free form but seems to be similar to the

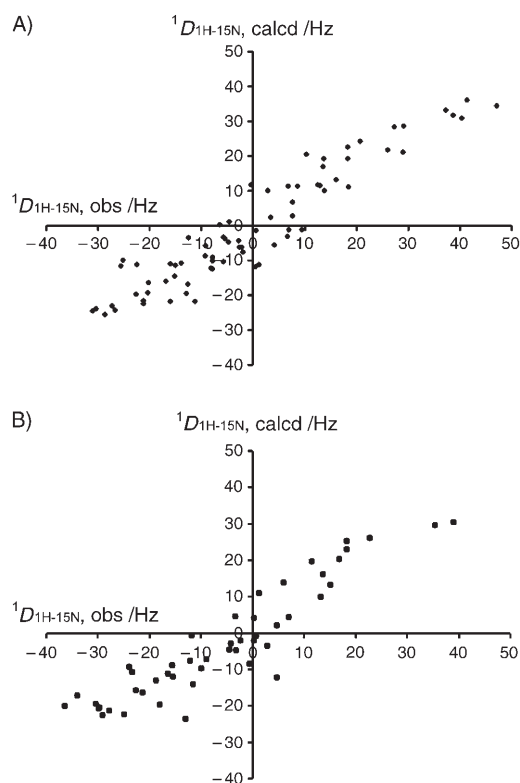


Figure 3. $^1D_{1H-15N}$ residual dipolar couplings for p38 α in its free and inhibitor-bound forms. A) RDC ensemble of the p38 α free form fitted to the crystal structure (PDB code: 1P38) with a correlation factor of 0.94 ($Q=0.25$); B) RDC values of the inhibitor-bound form show a correlation factor of 0.92 ($Q=0.31$).

conformation found in a subgroup of complex crystal structures.^[14]

Specific observation of resonances in the hinge region is hampered by strong overlaps in the $^1H-^{15}N$ TROSY-HSQC spectrum. To bypass this difficulty, we produced three samples with sequentially labeled amino acid pairs in the hinge region, $^{13}C^{15}N$ His/ ^{15}N Leu, $^{13}C^{15}N$ Leu/ ^{15}N Met, and $^{13}C^{15}N$ Met/ ^{15}N Gly. $^1H-^{15}N$ TROSY-HSQC and 2D HNCOC spectra were recorded on these samples in the free and inhibitor-bound forms. With this approach, most of the resonances of residues from the inhibitor-binding regions were unambiguously assigned for the p38 α free form. Surprisingly, many of these amide and methyl group resonances disappeared upon binding because of line broadening (Figure 4). This reveals that these regions are undergoing motion on the intermediate-exchange timescale (μs -to- ms timescale) in the presence of the inhibitor. Affected regions are the glycine-rich loop (Gly33 to Val38), parts of the hydrophobic pocket I (Ile84 to Leu86), and the hinge region (Thr106 to Ala111). Thus, more than one conformation of the p38 α /SB203580 complex exists in solution. This is in strong contrast to the rigid complex observed in the crystal structure but still in agreement with the low-resolution average binding mode derived in previous NMR studies.^[10]

Our NMR data show that the ATP-binding site of this complex appears to be highly flexible after inhibitor binding, in contrast to the seeming rigidity of the crystal structure. The

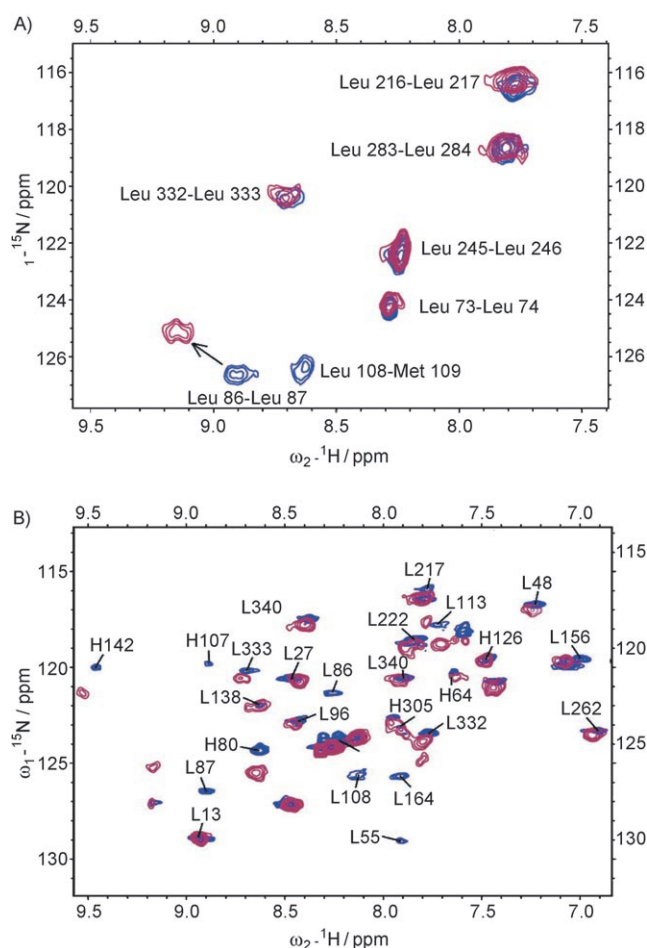


Figure 4. A) Overlay of the two-dimensional HNCO spectrum of the free form of p38 α (blue) with that of the p38 α /SB203580 complex (pink). The samples are ${}^{13}\text{C}/{}^{15}\text{N}$ Met labeled. Disappearing cross-peaks after addition of the inhibitor correspond to the Leu108 and Met109 residues from the hinge region; B) overlay of the two-dimensional ${}^1\text{H}-{}^{15}\text{N}$ TROSY-HSQC spectra acquired with selectively labeled ${}^{13}\text{C}/{}^{15}\text{N}$ His/ ${}^{15}\text{N}$ Leu samples (free form in blue, complex form in pink). Disappearing cross-peaks after addition of the inhibitor correspond to the His107 and Leu108 residues from the hinge region. H: histidine; L: leucine.

exact origin of the mobility in the p38 α /SB203580 complex remains unclear and will have to be investigated in more detail in the future. The flexibility originates not from the dipeptide flip of Gly110 and Ala111 between the crystal structure and the solution structure of the free form (described above), since these resonances are observed in the bound structure in solution.

Several interesting questions arise: does the inhibitor, according to its chemical nature, sample conformations of the kinase preexisting in solution? Are these different conformations associated with different intrinsic flexibilities of the binding regions or is the inhibitor itself undergoing dynamic changes (for example, ring flipping)? In both cases, it is most probable that, in addition to the structure observed in the crystal, at least one additional conformation exists, with these two (or more) conformations being in exchange on a micro- to millisecond timescale. It could be a challenging goal to

investigate whether this dynamic behavior is a common feature of p38 α /inhibitor complexes or whether it is specific to the SB203580 inhibitor. The determination of the additional conformations would give important information concerning efficient selectivity in drug design. Studies on other kinase/inhibitor complexes are under way to explore whether these results constitute a new paradigm for such complexes.

Experimental Section

Sample preparation: Single amino acid labeling and purification of human p38 α was carried out as published^[15,16] with minor modifications. All samples were concentrated to 1 mM. Inhibitors were prepared in $[\text{D}_6]$ dimethylsulfoxide ($[\text{D}_6]$ DMSO) at a concentration of 50 mM and added to the protein sample in a 2:1 ratio.

NMR spectroscopy: NMR experiments were carried out at 298 K on Bruker Avance spectrometers operating at 600 and 800 MHz and equipped with z -gradient cryoprobes. All spectra were processed by using the NMRPipe/NMRDraw^[17] and Xwinnmr (Bruker) software. Spectra were analyzed with the Sparky^[18] and CARRA^[19] software. ${}^1\text{H}$, ${}^{15}\text{N}$, and ${}^{13}\text{C}$ assignments of p38 α were taken from the Biological Magnetic Resonance Bank (BMRB) entry bmr6468.^[9] Backbone amide resonances of the p38 α /SB203580 complexes were assigned by using ${}^1\text{H}-{}^{15}\text{N}$ TROSY-HSQC, TROSY-HNCA, TROSY-HNCO, and ${}^1\text{H}-{}^{15}\text{N}$ HSQC-NOESY spectra recorded on a triple-labeled sample (${}^2\text{H}$, ${}^{13}\text{C}$, ${}^{15}\text{N}$) or on specifically labeled samples. RDC values were measured on a double-labeled sample (${}^2\text{H}$, ${}^{15}\text{N}$) in a partially aligned medium by using the bacteriophage Pf1 (Profos) at a concentration of 20 mg mL⁻¹ at pH 6, thereby providing a splitting of 12.76 Hz for the D_2O signal. One-bond ${}^1J_{\text{H}-{}^{15}\text{N}}$ and residual ${}^1D_{\text{H}-{}^{15}\text{N}}$ couplings were measured from ${}^1\text{H}-{}^{15}\text{N}$ HSQC and ${}^1\text{H}-{}^{15}\text{N}$ TROSY-HSQC spectra.

Received: December 7, 2007

Revised: January 17, 2008

Published online: April 3, 2008

Keywords: conformation analysis · inhibitors · kinases · NMR spectroscopy · protein dynamics

- [1] J. C. Lee, J. T. Laydon, P. C. McDonnell, T. F. Gallagher, S. Kumar, D. Green, D. McNulty, M. J. Blumenthal, J. R. Heys, S. W. Landvatter, J. E. Strickler, M. M. McLaughlin, I. R. Siemens, S. M. Fisher, G. P. Livi, J. R. White, J. L. Adams, P. R. Young, *Nature* **1994**, 372, 739.
- [2] G. J. Zaman, M. M. van der Lee, J. J. Kok, R. L. Nelissen, E. E. Loomans, *Assay Drug Dev. Technol.* **2006**, 4, 411.
- [3] D. Casper, M. Bukhtiyarova, E. B. Springman, *Anal. Biochem.* **2004**, 325, 126.
- [4] Z. Wang, B. J. Canagarajah, J. C. Boehm, S. Kassisa, M. H. Cobb, P. R. Young, S. Abdel-Meguid, J. L. Adams, E. J. Goldsmith, *Structure* **1998**, 6, 1117.
- [5] K. P. Wilson, P. G. McCaffrey, K. Hsiao, S. Pazhanisamy, V. Galullo, G. W. Bemis, M. J. Fitzgibbon, P. R. Garon, M. A. Murcko, M. S. S. Su, *Chem. Biol.* **1997**, 4, 423.
- [6] C. Pargellis, L. Tong, L. Churchill, P. F. Cirillo, T. Gilmore, A. G. Graham, P. M. Grob, E. R. Hickey, N. Moss, S. Pav, J. Regan, *Nat. Struct. Biol.* **2002**, 9, 268.
- [7] A. Trejo, H. Arzeno, M. Browner, S. Chanda, S. Cheng, D. D. Comer, S. A. Dalrymple, P. Dunten, J. Lafargue, B. Lovejoy, J. Freire-Moar, J. Lim, J. McIntosh, J. Miller, E. Papp, D. Reuter, R. Roberts, F. Sanpablo, J. Saunders, K. Song, A. Villasenor, S. D. Warren, M. Welch, P. Weller, P. E. Whiteley, L. Zeng, D. M. Goldstein, *J. Med. Chem.* **2003**, 46, 4702.

- [8] A. Cuenda, J. Rouse, Y. N. Doza, R. Meier, P. Cohen, T. F. Gallagher, P. R. Young, J. C. Lee, *FEBS Lett.* **1995**, 364, 229.
- [9] M. Vogtherr, K. Saxena, S. Grimme, M. Betz, U. Schieborr, B. Pescatore, T. Langer, H. Schwalbe, *J. Biomol. NMR* **2005**, 32, 175.
- [10] M. Vogtherr, K. Saxena, S. Hoelder, S. Grimme, M. Betz, U. Schieborr, B. Pescatore, M. Robin, L. Delarbre, T. Langer, K. U. Wendt, H. Schwalbe, *Angew. Chem.* **2006**, 118, 1008; *Angew. Chem. Int. Ed.* **2006**, 45, 993.
- [11] G. Kontaxis, G. M. Clore, A. Bax, *J. Magn. Reson.* **2000**, 143, 184.
- [12] M. Zweckstetter, A. Bax, *J. Am. Chem. Soc.* **2000**, 122, 3791.
- [13] Z. Wang, P. C. Harkins, R. J. Ulevitch, J. Han, M. H. Cobb, E. J. Goldsmith, *Proc. Natl. Acad. Sci. USA* **1997**, 94, 2327.
- [14] C. E. Fitzgerald, S. B. Patel, J. W. Becker, P. M. Cameron, D. Zaller, V. B. Pikounis, S. J. O'Keefe, G. Scapin, *Nat. Struct. Biol.* **2003**, 10, 764.
- [15] D. M. Lemaster, F. M. Richards, *Biochemistry* **1988**, 27, 142.
- [16] M. Bukhtiyarova, K. Northrop, X. Chai, D. Casper, M. Karpusas, E. Springman, *Protein Expression Purif.* **2004**, 37, 154.
- [17] F. Delaglio, S. Grzesiek, G. W. Vuister, G. Zhu, J. Pfeifer, A. Bax, *J. Biomol. NMR* **1995**, 6, 277.
- [18] <http://www.cgl.ucsf.edu/home/sparky/>.
- [19] R. Keller, *The computer aided resonance assignment tutorial*, Cantina, Goldau, **2004**.