

A Genetically Encoded ^{19}F NMR Probe for Tyrosine Phosphorylation**

Fahui Li, Pan Shi, Jiasong Li, Fan Yang, Tianyuan Wang, Wei Zhang, Feng Gao, Wei Ding, Dong Li, Juan Li, Ying Xiong, Jinpeng Sun, Weimin Gong,* Changlin Tian,* and Jiangyun Wang*

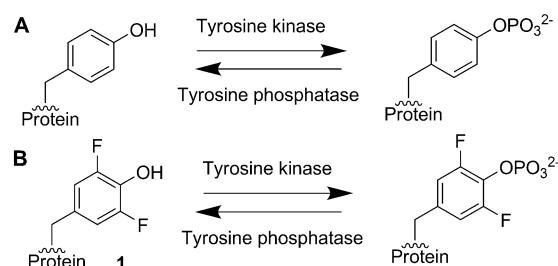
Dedicated to Professor Yufen Zhao on the occasion of her 65th birthday

Tyrosine phosphorylation is a pivotal post-translational modification (PTM) which regulates enzymatic activity, protein conformation, and protein–protein interactions.^[1] While the eukaryotic protein tyrosine kinases (PTKs) have been intensively studied in the past three decades because of their great importance in cellular signaling and diseases,^[2] the prokaryotic PTKs, which play ubiquitous roles in bacterial virulence, were found only recently, and their activation mechanism is controversial.^[3]

^{19}F NMR spectroscopy has recently emerged as a powerful tool for characterizing enzyme mechanisms and protein motions over a range of time scales, because of the high intrinsic sensitivity of fluorine, 100 % natural abundance of the NMR-active isotope, the absence of any natural background in proteins, and the exquisite sensitivity of the ^{19}F chemical shift to environment.^[4] Numerous chemical and biosynthetic methods have been developed for incorporating

fluorinated amino acids into proteins.^[5] Among these methods, the genetic code expansion technique has the unique advantages that fluorinated amino acids can be directly incorporated into specific sites of any protein of interest in living cells, and that the mutant protein can be easily obtained in milligram quantities. However, this powerful method has not been applied to investigate tyrosine phosphorylation.

Here we report the highly efficient genetic incorporation of the unnatural amino acid (UAA) 3,5-difluorotyrosine **1** (hereafter termed F2Y, Scheme 1), which mimics the



Scheme 1. Reversible tyrosine (A) or F2Y (B) phosphorylation and dephosphorylation catalyzed by PTK and PTP, respectively.

tyrosine substrate, into the activation loops (a-loop) of the bacterial PTK Etk and eukaryotic PTK Src in *E. coli*. While the presence of phosphotyrosine (pTyr) in the a-loop of many PTKs is well established, its exact role in regulating PTK activity and interacting with drug molecules is still not fully understood. A major limitation of our understanding is that such a PTM cannot be directly probed through traditional site-directed mutagenesis methods, and that pTyr is often susceptible to hydrolysis during the protein crystallization process. As a result, it remains very difficult to obtain structural information to elucidate how pTyr interacts with other residues or drug molecules. Through the genetic incorporation of **1** and using ^{19}F NMR spectroscopy, we provide for the first time direct evidence that Arg614 interacts with the a-loop residue pTyr574 to lock Etk in an active conformation. Substitution of Arg614 to Ala does not impair the autophosphorylation activity of Etk on residue Tyr574, but results in an inactive conformation with significantly decreased overall activity. We also show that dasatinib (a cancer drug targeting BCR/ABL and Src) binding to Src causes a significant conformation change in its a-loop bearing

[*] P. Shi,^[†] J. S. Li,^[†] D. Li, J. Li, Y. Xiong, C. L. Tian
School of Life Sciences, University of Science and
Technology of China, Hefei, Anhui, 230026 (China)
E-mail: cltian@ustc.edu.cn

F. H. Li,^[†] J. S. Li,^[†] T. Y. Wang, W. Zhang, F. Gao, W. Ding,
W. M. Gong, J. Y. Wang
Laboratory of Non-coding RNA, Institute of Biophysics
Chinese Academy of Sciences
15 Datun Road, Chaoyang District
Beijing, 100101 (China)
E-mail: wgong@ibp.ac.cn
jwang@ibp.ac.cn

F. Yang, J. P. Sun
Department of Physiology
Shandong University School of Medicine
Jinan 250012 (China)

P. Shi,^[†] C. L. Tian
High Magnetic Field Laboratory
Chinese Academy of Sciences
Hefei, Anhui, 230031 (China)

[†] These authors contributed equally to this work.

[**] We gratefully acknowledge the Major State Basic Research Program of China (grant numbers 2010CB912301 and 2009CB825505), the National Science Foundation of China (grant numbers 90913022, 31000364, 31170817, and 31100563), the CAS grant (grant number KSCX2-EW-G-7) to J.W., F.L., C.T., and Y.X. We thank Professors Zongchao Jia and Jingxuan Pan for helpful discussion.

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/ange.201300463>.

pTyr416. These insights cannot be obtained by conventional methods including X-ray diffraction (XRD) or mass spectrometry (MS).^[3] Our new method should be broadly applicable to the investigation of the tyrosine phosphorylation mechanism, protein–protein interactions mediated by tyrosine phosphorylation, and drug–PTK interactions.

Our investigation started from the synthesis of F2Y **1** and *o*-phospho-3,5-difluorotyrosine (pF2Y), following established methods.^[1b,5] The UAA F2Y was selected as a surrogate substrate of PTK, since it has been previously demonstrated that peptides containing F2Y showed similar efficiency as a PTK substrate compared with the corresponding tyrosine-containing peptide.^[6] We found that the ¹⁹F chemical shifts of F2Y and pF2Y were at $\delta = -133.1$ ppm and $\delta = -126.7$ ppm, respectively (see Figure S1 in the Supporting Information), which allowed for convenient differentiation of the F2Y and pF2Y ¹⁹F NMR signals.

To selectively incorporate F2Y at defined sites in proteins, a mutant *M. jannaschii* tyrosyl amber suppressor tRNA(*Mj*tRNA_{Tyr}^{CUA})/tyrosyl-tRNA synthetase (*Mj*TyrRS) pair was evolved that uniquely specifies **1** in response to the TAG codon, as previously reported.^[5j,k] One *Mj*TyrRS clone emerged after positive–negative selections, which grew at 160 $\mu\text{g mL}^{-1}$ of chloramphenicol in the presence of 1 mM **1**, but only at 20 $\mu\text{g mL}^{-1}$ chloramphenicol in its absence, and was named F2YRS (Tyr32Arg, Leu65Tyr, His70Gly, Phe108Asn, Gln109Cys, Asp158Asn, Leu162Ser).

To unravel the structural basis for the selective recognition of F2Y by F2YRS, we solved the crystal structures of F2YRS, in the presence and absence of F2Y. Since the phenol group of the F2Y substrate has a pK_a of 6.8,^[5e] F2Y is likely to be present in an anionic state, and binds favorably to the positively charged residue Arg32. One of the guanidine nitrogen atoms of Arg32 is involved in hydrogen-bonding interactions with the phenolic oxygen atom of F2Y. The amide nitrogen atom of Asn158 is located 3.4 Å away from one of the fluorine atoms in F2Y, and one of the terminal guanidine nitrogen atoms of Arg32 is situated 3.5 Å away from the other fluorine atom in F2Y (Figure 1 A). While these distances are too far away for hydrogen bonding, relatively strong dipolar interactions between the fluorine atoms and amide/guanidine groups may be present.^[7] Our observation is consistent with previous studies that organic fluorine is only a weak hydrogen-bond acceptor.^[8] We then superimposed the apo and holo F2YRS structures. As shown in Figure 1 B, most of the side-chain atoms in the F2YRS-active site make only small movements (less than 1 Å) to accommodate the F2Y substrate, which indicates a pre-organized active site. This feature may be important for maximal substrate binding because of the minimization of entropic loss, and the high catalytic efficiency of F2YRS. To gain further insight on how F2YRS discriminates against the tyrosine substrate, we performed structure alignment of F2YRS and the wt *Mj*TyrRS. As shown in Figure S2, the backbone of F2YRS and *Mj*TyrRS can be superimposed, and the tyrosine ligand occupies a similar position as F2Y. If F2YRS binds a tyrosine in its active site, the tyrosine ligand will displace some water molecules, decrease the local dielectric constant, and increase the free-energy of the positively charged residue Arg32. Since

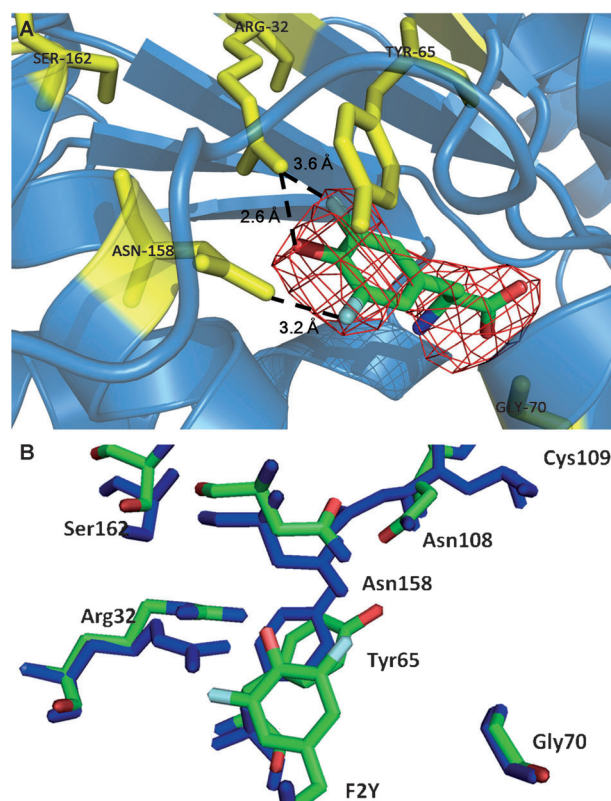


Figure 1. a) Structure of F2YRS with F2Y bound in the active site; the Fo-Fc electron density map of F2Y was contoured at 2.7 σ . b) Superposition of the active sites of holo F2YRS (green) and apo F2YRS (blue). Nitrogen atoms are shown in blue, oxygen atoms are shown in red, and fluorine atoms are shown in cyan.

the tyrosine ligand is likely to be present in a charge-neutral state, as the pK_a of the phenol group is 10.2, the ligand will not be able to stabilize the positive charge of Arg32 as F2Y does.

To determine whether F2YRS can facilitate the incorporation of F2Y into proteins with high efficiency and fidelity, we performed in vitro amino-acylation assays. As Figure 2 A shows, F2YRS only charged F2Y, but not tyrosine, to tRNA_{Tyr}^{CUA}. The F2YRS was then cloned into pEVOL plasmid as described^[5k] for enhanced expression of proteins containing F2Y. An amber stop codon was substituted for Tyr151 in the green fluorescent protein (GFP). Protein production was carried out in *E. coli* in the presence of F2YRS, *Mj*tRNA_{Tyr}^{CUA}, and 0.5 mM F2Y, or in the absence of F2Y as a negative control. Analysis of the purified GFP-151-F2Y by SDS-PAGE showed that full-length GFP was expressed only in the presence of F2Y (Figure 2 B), indicating that F2YRS was specifically active for F2Y but inactive for any natural amino acids. ESI-MS analysis of the GFP-151-F2Y mutant gave an observed average mass of 27746.0 Da, in agreement with the calculated mass of 27746.0 Da (Figure 2 C and D). The yield for mutant GFP was 60 mg L^{-1} . For comparison, the yield of wild-type GFP was 100 mg L^{-1} . This corresponded to a UAG codon suppression efficiency of 60 %. To the best of our knowledge, this is the highest UAG codon suppression efficiency ever reported for UAA incorporation.

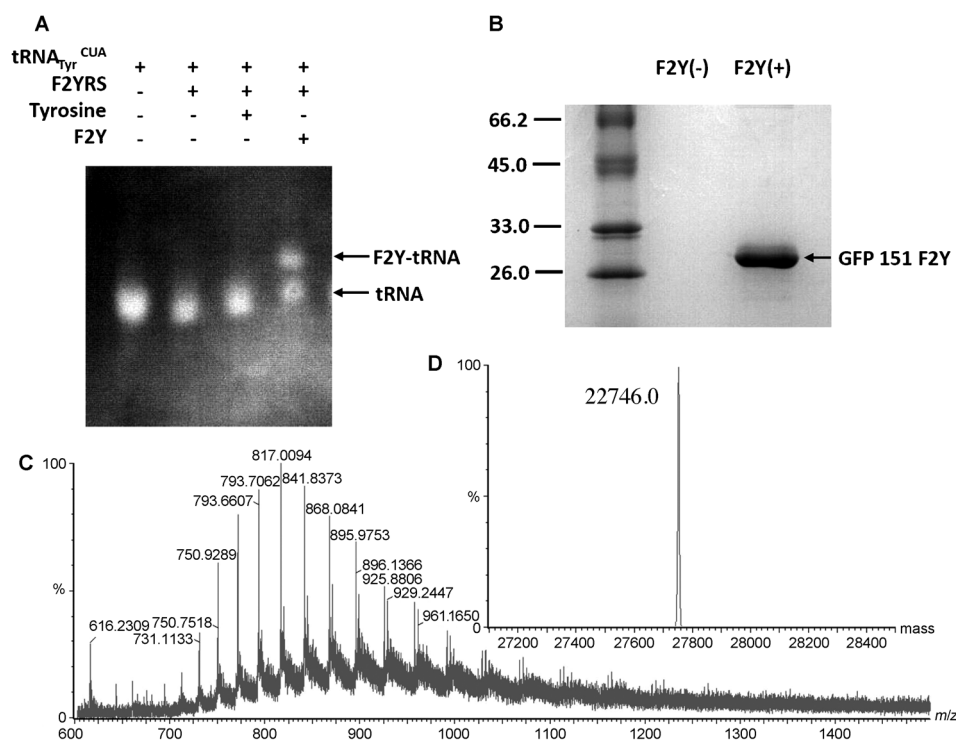


Figure 2. A) In vitro amino-acylation of tRNA_{Tyr}^{CUA} by F2YRS. B) Coomassie-stained SDS-PAGE of TAG151 mutant GFP (indicated by a black arrow) expression in the presence and absence of 0.5 mM F2Y. C) ESI-MS spectra of the GFP-TAG151-F2Y mutant. D) Deconvoluted spectrum. Expected mass: 27 746 Da, found: 27 746 Da.

This high efficiency is essential for NMR experiments since a large amount of sample is required, and eukaryotic PTK can be produced with only relatively low yield in *E. coli*.^[3g]

Etk is an inner-membrane PTK in gram-negative bacteria, which plays vital roles in regulating the polymerization and transport of virulence-determining capsular polysaccharide (CPS).^[2] The cytoplasmic domain of *E. coli* Etk (residues 451–726) contains a conserved Tyr574 in its a-loop. Through structural and mutational analysis, it was proposed that Etk activation requires Tyr574 auto-phosphorylation. The a-loop phosphorylation is also required for the activation of many eukaryotic PTK.^[9] As shown in Figure 3, in the inactive state, the Tyr574 side chain is situated in close proximity to the ADP substrate, blocking the substrate peptide from accessing the Etk active site. Once Tyr574 is phosphorylated, previously reported structural models suggested that it moves away from the catalytic center, binds to Arg614, and adopts a conformation that allows for substrate binding and catalysis.^[3g] However, phosphorylated Tyr574 was not observed in a recently reported crystal structure,^[3g] because of rapid dephosphorylation in the XRD sample preparation process.^[2] Indeed, it is well known that pTyr is less stable in phosphoamino acid analysis than pSer/pThr in general.^[10]

To verify if the a-loop Tyr574 is indeed phosphorylated as previously proposed, we expressed the Etk C-terminal cytoplasmic domain (residues 451–726) bearing F2Y at position 574 (termed Etk-574-F2Y) in *E. coli*, using pEVOL-F2YRS and pET28c-Etk574TAG plasmids. The yield of Etk-574-F2Y mutant was 12.5 mg L⁻¹. In comparison,

the yield of wt Etk was 20 mg L⁻¹ (Figure 4A). Tryptic digestion and tandem mass spectrometry confirmed that F2Y was selectively incorporated in the 574 position (Figure S3). Etk-574-F2Y auto-phosphorylation or dephosphorylation was then quantified using ¹⁹F solid-state NMR spectroscopy^[11] and western blot assays. An aliquot of each sample was saved for western blot analysis, and the remaining samples were frozen at -40°C, lyophilized, and subjected to solid-state NMR spectra acquisition. As shown in Figure 4C, the Etk-574-F2Y sample which was allowed to auto-phosphorylate gave rise to two ¹⁹F NMR signals at -122.3 ppm and -134.5 ppm, respectively. Upon PTP1B treatment, however, the peak at -122.3 ppm disappeared, whereas the peak at -134.5 ppm remained intact (Figure S4). These results indi-

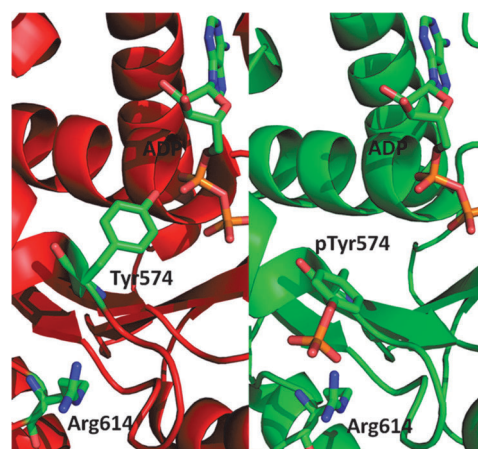


Figure 3. Left: Structure of *E. coli* PTK Etk (pdb code 3CIO). Right: Structure model of Etk, bearing a pTyr574 in its a-loop.

cate that the peak at -122.3 ppm corresponds to pF2Y at position 574. Consistent with these results, the pTyr signals in Etk-574-F2Y disappeared after PTP1B treatment, in a western-blot assay using the anti-pTyr antibody. Notably, the NMR signal intensity of pF2Y was only 3.8 % in comparison to that of F2Y, indicating that only a small fraction of tyrosine was phosphorylated in the a-loop of Etk. These results are consistent with previous reports that phosphorylation of the a-loop tyrosine in bacterial PTK is very low but sufficient to elicit extensive inter-phosphorylation.^[3e] Based on the Etk

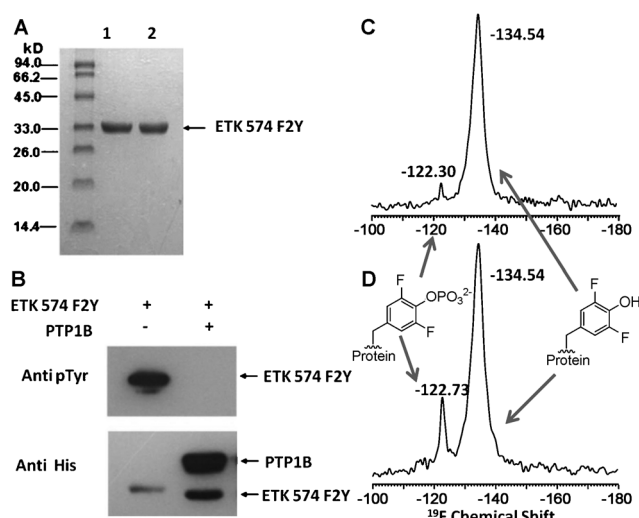


Figure 4. a) SDS-PAGE of wt Etk (lane 1) and Etk-574-F2Y (lane 2). b) Western blot experiment using an anti-pTyr antibody showed that phosphorylated Etk-574-F2Y can be efficiently dephosphorylated by PTP1B. c) ¹⁹F solid-state NMR spectrum of Etk-574-F2Y. Chemical shifts are referenced to trifluoroacetic acid (TFA) as an internal standard. d) ¹⁹F solid-state NMR spectrum of Etk-574-F2Y-R614A.

crystal structure and mutagenesis studies which showed that the Arg614Ala mutant has a much decreased overall catalytic activity,^[3g] a molecular model was constructed to suggest that Arg614 interacts with pTyr574 to unblock the active site, allowing for peptide and ATP substrate to bind (Figure 3). However, no direct evidence was provided to support this model.^[3g] To directly test this hypothesis, we expressed the mutant Etk-574-F2Y-R614A, and again allowed it to auto-phosphorylate in the same condition as described above. ¹⁹F solid-state NMR spectroscopy revealed that the pF2Y NMR signal shifted to -122.73 ppm, which was 0.43 ppm downfield compared to that of Etk-574-F2Y, suggesting that Arg614 does interact directly with pTyr574. Interestingly, the pF2Y/F2Y signal intensity ratio was 10.8% in Etk-574-F2Y-R614A, significantly larger than that of Etk-574-F2Y. These results suggest that while pTyr574 binding to Arg614 render the active site accessible to substrates, substitution of Arg614 to Ala does not impair the autophosphorylation activity of Etk on residue Tyr574. Instead, the Arg614Ala mutation may cause pTyr574 to bind to an alternative residue Arg572 (Figure S5), occluding the ATP/ADP substrates from binding to the active site, resulting in a closed and inactive conformation which is also less prone to undergo the reverse pTyr574 dephosphorylation reaction. It was previously shown that pF2Y undergoes dephosphorylation reaction 1.5 to 2 folds faster than that of pTyr,^[6] therefore the pF2Y/F2Y ratios observed in our experiments are likely underestimates of pTyr/Tyr ratios. Nevertheless, there is currently no reliable method available for quantifying pTyr/Tyr ratios, and pF2Y/F2Y ratios observed in different mutants can provide valuable mechanistic insights on how each mutation affects the pTyr/Tyr equilibrium. Interestingly, the ¹⁹F signal arising from the phosphorylated protein is significantly sharper than the ¹⁹F

signal of the unphosphorylated protein. This is due to different conformational flexibility of the two forms.

Since there is currently strong interest in using ¹⁹F NMR spectroscopy to investigate drug–protein interactions,^[4] we then verified if our method is applicable for detecting the interaction between dasatinib and c-Src, a key enzyme in cancer formation.^[14,3j–l] Src catalytic activity requires phosphorylation of Tyr416 in the a-loop. If Tyr416 is not phosphorylated, the a-loop adopts an alpha-helical, ordered but inhibitory conformation (Figure S6), blocking the substrate-binding site. Phosphorylation of Tyr416 stabilizes the a-loop far away from the active site, opening it up for catalytic turnover.^[3i–n] While a complex structure of dasatinib and c-Src was recently reported,^[3k] its a-loop appeared disordered (Figure S6), and it is not known if dasatinib binding to c-Src can cause conformation changes in its a-loop. To directly test this, we expressed the chicken c-Src bearing F2Y at position 416 (termed Src-416-F2Y), as previously described.^[3n] The conformation change of Src-416-F2Y auto-phosphorylation was detected by ¹⁹F solid-state NMR spectroscopy in the presence and absence of dasatinib. As Figure S6 shows, in the absence of dasatinib, the Src-416-F2Y sample gave rise to a ¹⁹F NMR signal at -125.1 ppm, indicating that F2Y at the 416 position was mostly phosphorylated. In the presence of dasatinib, the ¹⁹F NMR signal shifted to -122.4 ppm, indicating that dasatinib binding to Src triggers a significant conformation change in its a-loop, resulting in a 2.7 ppm upfield shift of the 416-pF2Y signal. Our results are consistent with previous studies which showed that dasatinib binds to the activated Abl and Src family PTKs,^[3j–l] and may provide valuable insights for designing better PTK inhibitors.^[14]

In conclusion, we have demonstrated the highly efficient genetic incorporation of the UAA F2Y in *E. coli*, and its use as a ¹⁹F NMR probe for tyrosine phosphorylation. We solved the structures of F2YRS in the presence and absence of F2Y, and unraveled the structural basis for the selective recognition of F2Y by F2YRS. Our new method for probing tyrosine phosphorylation has numerous advantages versus traditional strategies. First, tyrosine phosphorylation at a specific site in intact proteins can be detected and quantified. By contrast, peptide substrates cannot recapitulate distal interactions between PTKs and substrate proteins,^[12] which are often essential for activity. Second, samples can be frozen at any desired time point, followed by ¹⁹F NMR spectra acquisition. This allows for accurate phosphorylation quantification even in complex protein samples, and quantitative measurement of the *K_M* and *k_{cat}* of phosphorylation reactions.^[4j] Third, single-site ¹⁹F incorporation and NMR relaxation analysis can be conveniently applied to illustrate protein dynamic properties, because of the high sensitivity of ¹⁹F chemical shift anisotropy (CSA) to environment and absence of ¹⁹F–¹⁹F direct coupling. Further NMR studies may help to reveal the motions of eukaryotic and prokaryotic PTK in the active and inactive states, and dynamic exchange between these states.^[13] While a relatively large amount of protein is needed, our method has unique advantages over others, since it allows for the study of dynamic conformation changes in an intact protein, over a range of time scales. This sensitive, selective, and robust method for monitoring PTK activation and activity should

also provide a valuable tool to screen for novel inhibitors targeted for specific PTK/substrate protein interactions.^[14]

Received: January 18, 2013

Published online: February 28, 2013

Keywords: NMR spectroscopy · protein–protein interactions · proteins · tyrosine kinase · tyrosine phosphorylation

- [1] a) T. Hunter, *Curr. Opin. Cell Biol.* **2009**, *21*, 140–146; b) W. Eckhart, M. A. Hutchinson, T. Hunter, *Cell* **1979**, *18*, 925–933; c) R. Karisch, M. Fernandez, P. Taylor, C. Virtanen, J. R. St-Germain, L. L. Jin, I. S. Harris, J. Mori, T. W. Mak, Y. A. Senis, A. Ostman, M. F. Moran, B. G. Neel, *Cell* **2011**, *146*, 826–840.
- [2] a) A. C. Dar, K. M. Shokat, *Annu. Rev. Biochem.* **2011**, *80*, 769–795; b) J. A. Endicott, M. E. M. Noble, L. N. Johnson, *Annu. Rev. Biochem.* **2012**, *81*, 587–613; c) J. M. Zhang, P. L. Yang, N. S. Gray, *Nat. Rev. Cancer* **2009**, *9*, 28–39; d) N. S. Gray, L. Wodicka, A. Thunnissen, T. C. Norman, S. J. Kwon, F. H. Espinoza, D. O. Morgan, G. Barnes, S. LeClerc, L. Meijer, S. H. Kim, D. J. Lockhart, P. G. Schultz, *Science* **1998**, *281*, 533–538; e) N. Jura, N. F. Endres, K. Engel, S. Deindl, R. Das, M. H. Lamers, D. E. Wemmer, X. W. Zhang, J. Kuriyan, *Cell* **2009**, *137*, 1293–1307; f) M. A. Seeliger, M. Young, M. N. Henderson, P. Pellicena, D. S. King, A. M. Falick, J. Kuriyan, *Protein Sci.* **2005**, *14*, 3135–3139; g) V. K. Lacey, A. R. Parrish, S. Han, Z. Shen, S. P. Briggs, Y. Ma, L. Wang, *Angew. Chem.* **2011**, *123*, 8851–8855; *Angew. Chem. Int. Ed.* **2011**, *50*, 8692–8696.
- [3] a) C. Grangeasse, S. Nessler, I. Mijakovic, *Philos. Trans. R. Soc. London Ser. B* **2012**, *367*, 2640–2655; b) D. C. Lee, Z. C. Jia, *Trends Biochem. Sci.* **2009**, *34*, 351–357; c) F. Jadeau, C. Grangeasse, L. Shi, I. Mijakovic, G. Deleage, C. Combet, *Nucleic Acids Res.* **2012**, *40*, D321–D324; d) C. Grangeasse, A. J. Cozzzone, J. Deutscher, I. Mijakovic, *Trends Biochem. Sci.* **2007**, *32*, 86–94; e) C. Grangeasse, P. Doublet, A. J. Cozzzone, *J. Biol. Chem.* **2002**, *277*, 7127–7135; f) C. Vincent, P. Doublet, C. Grangeasse, E. Vaganay, A. J. Cozzzone, B. Duclos, *J. Bacteriol.* **1999**, *181*, 3472–3477; g) D. C. Lee, J. M. Zheng, Y. M. She, Z. C. Jia, *EMBO J.* **2008**, *27*, 1758–1766; h) T. Lu, H. W. Tan, D. Lee, G. J. Chen, Z. C. Jia, *Biochemistry* **2009**, *48*, 7986–7995; i) W. Xu, A. Doshi, M. Lei, M. J. Eck, S. C. Harrison, *Mol. Cell* **1999**, *3*, 629–638; j) L. J. Lombardo, F. Y. Lee, P. Chen, D. Norris, J. C. Barrish, K. Behnia, S. Castaneda, L. A. Cornelius, J. Das, A. M. Doweyko, C. Fairchild, J. T. Hunt, I. Inigo, K. Johnston, A. Kamath, D. Kan, H. Klei, P. Marathe, S. Pang, R. Peterson, S. Pitt, G. L. Schieven, R. J. Schmidt, J. Tokarski, M. L. Wen, J. Wityak, R. M. Borzilleri, *J. Med. Chem.* **2004**, *47*, 6658–6661; k) M. Getlik, C. Grutter, J. R. Simard, S. Kluter, M. Rabiller, H. B. Rode, A. Robubi, D. Rauh, *J. Med. Chem.* **2009**, *52*, 3915–3926; l) J. S. Tokarski, J. A. Newitt, C. Y. Chang, J. D. Cheng, M. Wittekind, S. E. Kiefer, K. Kish, F. Y. Lee, R. Borzilleri, L. J. Lombardo, D. Xie, Y. Zhang, H. E. Klei, *Cancer Res.* **2006**, *66*, 5790–5797; m) L. N. Johnson, *Q. Rev. Biophys.* **2009**, *42*, 1–40; n) M. A. Seeliger, B. Nagar, F. Frank, X. Cao, M. N. Henderson, J. Kuriyan, *Structure* **2007**, *15*, 299–311.
- [4] a) J. J. Liu, R. Horst, V. Katritch, R. C. Stevens, K. Wuthrich, *Science* **2012**, *335*, 1106–1110; b) M. A. Danielson, J. J. Falke, *Annu. Rev. Biophys. Biomol. Struct.* **1996**, *25*, 163–195; c) P. Shi, D. Li, J. Li, H. W. Chen, F. M. Wu, Y. Xiong, C. L. Tian, *J. Phys. Chem. Lett.* **2012**, *3*, 34–37; d) P. Shi, H. Wang, Z. Y. Xi, C. W. Shi, Y. Xiong, C. L. Tian, *Protein Sci.* **2011**, *20*, 224–228; e) S. M. Elvington, C. W. Liu, M. C. Maduke, *EMBO J.* **2009**, *28*, 3090–3102; f) J. L. Kiteviski-LeBlanc, R. S. Prosser, *Prog. Nucl. Magn. Reson. Spectrosc.* **2012**, *62*, 1–33; g) C. G. Li, G. F. Wang, Y. Q. Wang, R. Creager-Allen, E. A. Lutz, H. Scronce, K. M. Slade, R. A. S. Ruf, R. A. Mehl, G. J. Pielak, *J. Am. Chem. Soc.* **2010**, *132*, 321–327; h) M. Ieronimo, S. Afonin, K. Koch, M. Berditsch, P. Wadhvani, A. S. Ulrich, *J. Am. Chem. Soc.* **2010**, *132*, 8822–8824; i) C. Dalvit, E. Ardini, M. Flocco, G. P. Fogliatto, N. Mongelli, M. Veronesi, *J. Am. Chem. Soc.* **2003**, *125*, 14620–14625.
- [5] a) C. J. Pace, H. Zheng, R. Mylvaganam, D. Kim, J. M. Gao, *Angew. Chem.* **2012**, *124*, 107–111; *Angew. Chem. Int. Ed.* **2012**, *51*, 103–107; b) M. Salwiczek, E. K. Nyakatura, U. I. M. Gerling, S. J. Ye, B. Koks, *Chem. Soc. Rev.* **2012**, *41*, 2135–2171; c) J. C. Jackson, J. T. Hammill, R. A. Mehl, *J. Am. Chem. Soc.* **2007**, *129*, 1160–1166; d) E. C. Minnihan, D. D. Young, P. G. Schultz, J. Stubbe, *J. Am. Chem. Soc.* **2011**, *133*, 15942–15945; e) M. R. Seyedsayamdost, S. Y. Reece, D. G. Nocera, J. Stubbe, *J. Am. Chem. Soc.* **2006**, *128*, 1569–1579; f) Y. Takaoka, Y. Sun, S. Tsukiji, I. Hamachi, *Chem. Sci.* **2011**, *2*, 511–520; g) H. Zheng, J. M. Gao, *Angew. Chem.* **2010**, *122*, 8817–8821; *Angew. Chem. Int. Ed.* **2010**, *49*, 8635–8639; h) D. Maisch, P. Wadhvani, S. Afonin, C. Bottcher, B. Koks, A. S. Ulrich, *J. Am. Chem. Soc.* **2009**, *131*, 15596–15597; i) L. Wang, P. G. Schultz, *Angew. Chem.* **2005**, *117*, 34–68; *Angew. Chem. Int. Ed.* **2005**, *44*, 34–66; j) X. H. Liu, Y. Yu, C. Hu, W. Zhang, Y. Lu, J. Y. Wang, *Angew. Chem.* **2012**, *124*, 4388–4392; *Angew. Chem. Int. Ed.* **2012**, *51*, 4312–4316; k) X. Liu, J. Li, J. Dong, C. Hu, W. Gong, J. Y. Wang, *Angew. Chem.* **2012**, *124*, 10407–10411; *Angew. Chem. Int. Ed.* **2012**, *51*, 10261–10265; l) M. R. Seyedsayamdost, C. S. Yee, J. Stubbe, *Nat. Protoc.* **2007**, *2*, 1225–1235.
- [6] K. Kim, P. A. Cole, *J. Am. Chem. Soc.* **1998**, *120*, 6851–6858.
- [7] F. R. Fischer, W. B. Schweizer, F. Diederich, *Angew. Chem.* **2007**, *119*, 8418–8421; *Angew. Chem. Int. Ed.* **2007**, *46*, 8270–8273.
- [8] J. D. Dunitz, R. Taylor, *Chem. Eur. J.* **1997**, *3*, 89–98.
- [9] a) P. A. Lochhead, G. Sibbet, N. Morrice, V. Clegdon, *Cell* **2005**, *121*, 925–936; b) H. Yamaguchi, W. A. Hendrickson, *Nature* **1996**, *384*, 484–489.
- [10] J. V. Olsen, B. Blagoev, F. Gnadt, B. Macek, C. Kumar, P. Mortensen, M. Mann, *Cell* **2006**, *127*, 635–648.
- [11] a) M. Renault, S. Pawsey, M. P. Bos, E. J. Koers, D. Nand, R. Tommassen-van Boxtel, M. Rosay, J. Tommassen, W. E. Maas, M. Baldus, *Angew. Chem.* **2012**, *124*, 3053–3056; *Angew. Chem. Int. Ed.* **2012**, *51*, 2998–3001; b) M. Renault, A. Cukkeman, M. Baldus, *Angew. Chem.* **2010**, *122*, 8524–8535; *Angew. Chem. Int. Ed.* **2010**, *49*, 8346–8357; c) R. Schneider, C. Ader, A. Lange, K. Giller, S. Hornig, O. Pongs, S. Becker, M. Baldus, *J. Am. Chem. Soc.* **2008**, *130*, 7427–7435; d) R. Witter, U. Sternberg, A. S. Ulrich, *J. Am. Chem. Soc.* **2006**, *128*, 2236–2243; e) M. Baldus, *Angew. Chem.* **2006**, *118*, 1204–1207; *Angew. Chem. Int. Ed.* **2006**, *45*, 1186–1188.
- [12] J. A. González-Vera, *Chem. Soc. Rev.* **2012**, *41*, 1652–1664.
- [13] a) J. S. Fraser, M. W. Clarkson, S. C. Degnan, R. Erion, D. Kern, T. Alber, *Nature* **2009**, *462*, 669–U149; b) K. A. Henzler-Wildman, V. Thai, M. Lei, M. Ott, M. Wolf-Watz, T. Fenn, E. Pozharski, M. A. Wilson, G. A. Petsko, M. Karplus, C. G. Hubner, D. Kern, *Nature* **2007**, *450*, 838–U813; c) B. F. Volkman, D. Lipson, D. E. Wemmer, D. Kern, *Science* **2001**, *291*, 2429–2433.
- [14] Z. A. Knight, H. Lin, K. M. Shokat, *Nat. Rev. Cancer* **2010**, *10*, 130–137.