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COMMUNICATION

Optimized bacterial expression and purification of the c-Src catalytic domain for solution NMR studies

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Abstract Progression of a host of human cancers is associated with elevated levels of expression and catalytic activity of the Src family of tyrosine kinases (SFKs), making them key therapeutic targets. Even with the availability of multiple crystal structures of active and inactive forms of the SFK catalytic domain (CD), a complete understanding of its catalytic regulation is unavailable. Also unavailable are atomic or near-atomic resolution information about their interactions, often weak or transient, with regulating phosphatases and downstream targets. Solution NMR, the biophysical method best suited to tackle this problem, was previously hindered by difficulties in bacterial expression and purification of sufficient quantities of soluble, properly folded protein for economically viable labeling with NMR-active isotopes. Through a choice of optimal constructs, co-expression with chaperones and optimization of the purification protocol, we have achieved the ability to bacterially produce large quantities of the isotopically-labeled CD of c-Src, the prototypical SFK, and of its activating Tyr-phosphorylated form. All constructs produce excellent spectra allowing solution NMR studies of this family in an efficient manner.

Keywords Non-receptor protein tyrosine kinases · Src-family kinases · *E. coli* expression and purification · Solution NMR methods

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Introduction

The nine members (c-Src, Blk, Fgr, Fyn, Hck, Lck, Lyn, Yes and Yrk) of the Src family of protein tyrosine kinases (SFK) constitute a class of non-receptor protein tyrosine kinases (NrPTK) that regulate many cellular events including cell proliferation, cytoskeleton alteration, differentiation, survival, adhesion, and migration, and play a central role in every aspect of the growth, differentiation and survival of higher eukaryotes (Courtneidge et al. 1993; Cooper and Howell 1993; Martin 2001). SFKs are 50–60 kDa proteins, which share a similar overall structure, and contain a catalytic domain (CD), that has a fold largely conserved across the protein kinome. In addition to the CD, SFKs contain the regulatory Src homology 3 (SH3) and Src homology 2 (SH2) domains (Cowan-Jacob 2006). The SFKs also contain a N-terminal domain with a lipidation motif important for membrane association (Resh 1994). The catalytic activity is wholly contained within the CD, and depends on the phosphorylation state of two key tyrosine residues: (1) activation at a tyrosine residue (Y_{act}, 416 in chicken c-Src) in the activation-loop in the CD; (2) suppression at a tyrosine residue (Y_{tail}, 527 in chicken c-Src) located in the regulatory C-terminal tail. Phosphorylation occurs at Y_{act} through an intermolecular autocatalytic mechanism leading to 10–100-fold increase in enzyme potency (Williams et al. 1998). Y_{tail} on the other hand, is phosphorylated by another family of

NrPTKs comprising two members—C-terminal Src kinase (Csk) (Okada et al. 1991) and Csk-homologous kinase (Chk) (Grgurevich et al. 1997). Phosphorylated Y_{tail} engages the SH2 domain in an intramolecular fashion leading to the downregulated state. Thus the phosphorylation of Y_{act} and Y_{tail} work in opposition—phosphorylation of the former leading to upregulation and that of the latter to downregulation of SFKs.

The transition between the fully deactivated and the fully activated states of SFKs involves multiple events, which may or may not be independent and the exact order of which are not yet clearly delineated (Boggon and Eck 2004). Some of these key events include—(1) phosphorylation of Y_{act}, (2) dephosphorylation of Y_{tail}, (3) disengagement of the C-terminal tail from the SH2 domain and (4) disengagement of the linker between the SH2 and catalytic domains from the SH3 domain. However, several studies strongly suggest that phosphorylation of Y_{act} in SFKs is sufficient for near-complete upregulation and for the regulatory components within the CD to attain the active state conformation (Sun et al. 1998; Donella-Deana et al. 1998). Thus, Y_{act} phosphorylation may be considered the central step in the activation of SFKs.

Multiple crystal structures of the active and inactive forms of representative members of the SFKs (Boggon and Eck 2004) and several in silico studies (Young et al. 2001; Banavali and Roux 2005, 2007) indicate substantial spatio-temporal changes in the relative orientation of the N-terminal and C-terminal lobes of the CD and in the key conserved regulatory regions—the hinge region between the lobes of the CD, the activation loop and a regulatory helix (helix αC) in the N-terminal lobe of the CD, among others. These changes appear to be key in the upregulation of kinase activity upon phosphorylation of Y_{act}. However, many important features of the activation pathway of the SFKs may not necessarily be evident from available crystal structures since they may not represent the endpoints of the activation pathways, but only those stable in crystal packing. Additionally, activation of enzymatic activity may not necessarily involve large changes in conformation that can be characterized by crystal structures alone, but involve changes in dynamics as seen in the case of the Eph receptor tyrosine kinase (Wiesner et al. 2006). The in silico studies may not have access to all timescales that are relevant in catalytic activation. Thus, solution state NMR studies would appear well suited to address the long-standing questions about the regulatory pathways involved in SFK activation. Notably, NMR has revealed novel regulatory pathways in other kinases including evidence of allosteric control as recently demonstrated in protein kinase A (PKA) (Masterson et al. 2008). Further, there are no available structures of any of the SFKs in complex with their downstream targets or with downregulating phosphatases. This is not surprising since these interactions are expected to be quite weak or transient, making crystallization of these complexes difficult. Here too, solution NMR provides an excellent method to identify key residues involved in protein–protein interactions through chemical shift mapping (Zuiderweg 2002) combined with improvements in the ability to study large protein–protein complexes (Riek et al. 2000; Sprangers and Kay 2007), especially when the structures of the individual interacting partners are available. Given these issues, solution NMR studies would be the biophysical technique of choice to answer these long-standing questions in the regulation and interactions of SFKs. However, the expression and purification of sufficient quantities of properly folded SFKs from bacterial expression systems to allow efficient isotope labeling, has been quite elusive, limiting the degree to which NMR could play a role in structural and dynamic studies of these signaling molecules. Here we provide a method that allows the bacterial expression and purification of c-Src CD both in wild-type and in a catalytically-compromised mutant form making detailed solution NMR studies of these medically relevant signaling proteins economically viable.

Materials and methods

The coding region for amino acids 248–533 (a kind gift from Dr. Alexander Shekhtman, SUNY Albany) of chicken c-Src K295M mutant ($SrcCD_{K295M}$) was amplified using the primers GGATCCAAACCCCAGACCCAGGGACTC and GTCGACCTATAGGTTCTCTCCAGGCTGGTACTG and sub-cloned into a H-MBP-3C vector (Ampicillin resistant) (Alexandrov et al. 2001) between the BamHI and SalI restriction sites. The pREP4-groESL (Kanamycin resistant) (Cole 1996) and the H-MBP-3C vector containing the c-Src constructs were introduced in the same expression system using two consecutive electroporations. With the first electroporation the pREP4-groESL was introduced and plated under Kanamycin selection. Electrocompetent BL21 cells containing the pREP4-groESL plasmid were prepared using standard protocols (the vector was transformed in commercially available electrocompetent BL21 cells, followed by growth in LB in the presence of 50 mg/L Kanamycin to an OD of 0.6, extensively washed with ice-cold water, and resuspended in a 10% glycerol solution upto 1/800th of the total volume). The H-MBP-3C vector was then introduced with the second electroporation, where the transformed cells were plated under both Ampicillin (100 mg/L) and Kanamycin (50 mg/L) selection. From these bacterial colonies, a 20 mL culture was grown overnight at 37°C in the presence of both Kanamycin and Ampicillin and used to inoculate 1 L of

fresh medium. These cells were grown using shaker flasks at 37°C to an $OD_{280} \sim 0.8$ and induced with 200 μM IPTG. After incubation at 37°C for 20 min, the cells were transferred to a 15°C shaker and grown for 14 h. These operations were performed using the appropriate M9 media containing $^{15}NH_4Cl$ and eventually [U-^{13}C]-glucose as the only nitrogen and carbon sources, respectively. For growth utilizing the algal lysate medium (Spectra-9 from Spectra Stable Isotopes), typically 1.5 g/L of labeled material was added to M9 medium, prepared in H_2O, containing 1 g/L of both $^{15}NH_4Cl$ and natural abundance ^{1}H-^{12}C-glucose. The harvested cells were resuspended in lysis buffer (20 mM Tris buffer, 0.5 M NaCl, 1% Triton X-100, pH 7.8, 40 mL addition for 1 L growth), passed twice through a French pressure cell (13,000 psi), centrifuged at 13,000 g (20 min) and finally loaded on a pre-equilibrated cobalt-chelating column. After elution with lysis buffer containing 300 mM imidazole, the fusion protein was dialyzed against the cleavage buffer (20 mM Tris, 150 mM NaCl, pH 7.8) and cleaved overnight with five units of α-thrombin per mg of fusion at 4°C, in the presence of 4 mM β-mercaptoethanol. The cleavage solution was then diluted in a low-ionic strength buffer (20 mM Tris, 5% glycerol, 1 mM DTT, 1 mM EDTA, pH 7.4) and loaded onto a Hi Trap Q-exchange column. $SrcCD_{K295M}$ was purified from the MBP fusion using a very shallow gradient (0–300 mM NaCl in 120 min). No more than 15 mg of the fusion protein was loaded on the column in a single run. $SrcCD_{K295M}$ eluted in two distinct peaks (corresponding to the unphosphorylated and singly-phosphorylated forms, see below) that were combined to yield a ~9.0 μM solution in about 20 mL (for each chromatographic purification run). The entire procedure yielded approximately 20–25 mg of purified $SrcCD_{K295M}$ per liter of aqueous M9.

The identity of the purified product was confirmed using SDS-PAGE and mass spectrometric analysis. NMR samples contained between 0.2 and 0.6 mM (prepared in 5 mm thin-walled NMR tubes susceptibility-matched with water, 300 μL total volume) protein in 20 mM bis–Tris, 200 mM NaCl, 200 mM PMSF, 1 mM DTT, 1 mM EDTA, 0–5% glycerol, 10% D_2O, pH 6.5 or 20 mM Tris, 150 mM NaCl, 1 mM DTT, 1 mM EDTA at pH 7.8 with 0–5% glycerol.

The vector encoding the wild-type c-Src catalytic domain ($SrcCD_{WT}$) was generated using the QuickChange kit (Stratagene) and the following primer: CACCAGAGTGGC CATAAAGACTCTGAAGCCCG and its reverse complement. The protocol for expression and purification was the same as that described above with an excess of the kinase inhibitor AP23464 (a kind gift of Dr. David Dalgarno of Ariad Pharmaceuticals) (Dalgarno et al. 2006) being added to the lysis buffer.

The yield of the Y_{act}-phosphorylated (Y416) form of $SrcCD_{K295M}$ (**p**$SrcCD_{K295M}$) was maximized by incubating the fusion protein obtained above (typically a mixture of $SrcCD_{K295M}$ and **p**$SrcCD_{K295M}$) at 4°C overnight with 5 mM ATP and 10 mM $MgCl_2$ with a catalytic amount of commercial wild-type, full-length, active c-Src (SIGMA, about 40 ng per mg of fusion) in 100 mM phosphate buffer containing 100 mM NaCl, pH 7.8. Similarly, in order to maximize our yield of $SrcCD_{K295M}$, we added commercially available alkaline phosphatase (New England Biolabs) directly to the cleavage reaction (pH 7.8) or to the elution peak (corresponding to **p**$SrcCD_{K295M}$, pH 7.4). Typically 4.0 units of phosphatase per mg of fusion were added to the solutions and followed by incubation overnight at 4°C.

Results and discussion

Recently, Vajpai et. al. (2008) have presented NMR studies on the CD of c-Abl expressed and ^{13}C, ^{15}N-labeled using baculovirus infected insect cells. However, this strategy is extremely cost-prohibitive and restricts the ability to deuterate or selectively label proteins especially at key side-chain positions e. g., ^{13}C,^{1}H-labeling at Ile, Leu and Val methyl positions (Tugarinov and Kay 2005). Loss of this ability severely compromises obtaining high-resolution structural and dynamic information on the SFKs. Clearly, bacterial expression and purification allows a host of labeling strategies in a cost-effective way. A strategy to bacterially express the CD of c-Src has been developed by Seeliger et al. (2005) using co-expression with YopH phosphatase to downregulate kinase activity and the resultant toxicity to the bacterial cells drawing from a successful approach utilized to express c-Src in *S. pombe* (Weijland et al. 1996). We, however, chose to use an alternative strategy pioneered by Cole and co-workers (Cole 1996).

A critical step before proceeding with optimization of the expression and purification of the CD of c-Src was a careful choice of construct. A relatively high expression level of proteins in properly folded, soluble form in *E. coli*, is required to make isotope labeling, which is necessary for NMR measurements, economically viable. This goal had so far been elusive for the wild-type c-Src CD (also see below) presumably due to the high levels of toxicity associated with its active state and proper folding. Thus, our first approach was to express a mutant with reduced enzymatic activity to prevent cytotoxicity. The first mutant species we decided to work with was the K295M point mutant (Wang et al. 2001), known to be of significantly lower catalytic competence. The particular mutant is compromised in its ability to bind ATP and has decreased kinase activity causing less toxicity to the cells. It has also been suggested that the K295 residue (located on helix αC)

participates in the formation of an ion-pair with E310 that helps stabilize the active state. While expressing full-length c-Src was a long-term goal, our initial choice of the length of construct was guided by the fact our immediate goal was to ascertain the functional dynamics and interactions specifically associated with activation resultant from phosphorylation of Y_{act}. As stated earlier, this single event has been shown to be central to the upregulation of the kinase activity of SFKs, even when Y_{tail} is in the phosphorylated state and presumably held in an assembled conformation by intramolecular interactions with the SH2 domain (Donella-Deana et al. 1998; Sun et al. 1998). Additionally, it has been shown that a portion of the linker between the SH2 and catalytic domains acts as a conformational switch facilitating the release of the SH2 and SH3 domains from the closed state conformation upon kinase activation (Banavali and Roux 2005). This behavior has been noticed in silico even when the SH2 and SH3 domains are absent. This region in c-Src includes conserved Trp (W260) and Asp (D258) residues. The motion of the sidechains of W260 was shown to be coupled to the rotation of the αC helix, one of the major conformational changes in the transition from the inactive to the active state conformation of the CD. Notably, mutation of W260 to Ala significantly enhances kinase activity even when the negative regulatory Y_{tail} is phosphorylated (Gonfloni et al. 1997; LaFevre-Bernt et al. 1998). Thus, in order to study the effects of phosphorylation of Y_{act} and include the regulatory influence of the linker between the SH2 and catalytic domains thereon, we chose a construct consisting of residues 248–533 (this construct encompasses the linker discussed earlier and includes the entire CD bearing the C-terminal tail including the negative regulatory Y_{tail}) in chicken c-Src for our studies.

We initially co-expressed the CD of the catalytically-compromised mutant of c-Src (K295M) with the groEL and groES chaperones. In our preliminary attempts to express and purify $SrcCD_{K295M}$ [using MBP (Alexandrov et al. 2001), gB1(Zhou et al. 2001) or Intein tags (Muir et al. 1998)] from minimal media, we obtained very small amounts of pure protein if co-expression with the groEL/groES chaperones was omitted, indicating that the suppression of enzymatic activity alone was not sufficient to improve the yield of properly folded, soluble protein. Thus, using a combination of the MBP solubility tag, co-expression with the groEL/groES chaperonins, bacterial growth at low temperature and low concentration of inducer we optimized a protocol to express and purify sufficient quantities of this protein per liter of M9 media (prepared in water), sufficient to prepare roughly six NMR samples. Altering any one of these factors resulted in up to ten-fold lower yields of pure protein. The His_6-tag introduced in the H-MBP-3C vector also proved useful to separate the monomeric form of the fusion protein from soluble aggregates that constituted a small but significant percentage of the lysate supernatant. While both forms were capable of binding amylose beads, only the desired monomeric form was retained by the cobalt-chelating resin.

During the purification process of $SrcCD_{K295M}$, we made the observation that a significant fraction of the purified protein was tyrosine-phosphorylated at Y_{act} (416, see below). and it was possible to separate the two components using ion-exchange chromatography. The ratio of the phosphorylated to unphosphorylated protein was dependent on the expression medium. In particular, for M9 media, an equal amount of the two states was obtained for low glucose concentration (2.5 g/L) while a 5:1 phosphorylated/unphosphorylated ratio, or higher, was typically observed using 5 g/L of glucose. A slightly lower ratio (3:1) was observed in LB. Additionally, equal amounts of the two states was obtained in both M9 and LB media if the chaperones were not co-expressed.

This procedure allowed us to generate $SrcCD_{K295M}$ both in the pure unphosphorylated form and that selectively phosphorylated on Tyr_{act} (**p**$SrcCD_{K295M}$). The identity of the phosphorylated form was confirmed using immunoblotting with anti-phosphoTyr antibody (from Zymed) before and after treatment with either alkaline phosphatase or Lyp phosphatase. Detectable binding was seen only for phosphorylated $SrcCD_{K295M}$. The non-specific alkaline phosphatase removes the phosphate moiety from all residues, while Lyp phosphatase is known to specifically dephosphorylate the phosphorylated Y_{act} [394 in Lck (Wu et al. 2006)]. The activation loops in Lck and c-Src are identical, it is therefore expected that Lyp would be specific to Y_{act} (416 in Src). The identity of the **p**$SrcCD_{K295M}$ was further confirmed by mass-spectrometric analysis.

Our preliminary NMR data (Fig. 1) shows that the two forms ($SrcCD_{K295M}$ and **p**$SrcCD_{K295M}$) produce well-resolved spectra suitable for the characterization of structural and dynamic changes upon activating phosphorylation. We found that 88% of the peaks were seen/resolved for a ^{15}N, ^{1}H HSQC at 900 MHz even for fully-protonated $SrcCD_{K295M}$ (273 non-proline amide groups, 7 Asn, 14 Gln NH_2 moieties and 6 Trp indole NH groups. Lys and Arg sidechain NH's not reported; total expected 321, seen 281).

Prior to the availability of complete assignments for the CD of c-Src, it was important to assess whether key regulatory elements within the c-Src CD were observable by NMR, most notably the activation loop. In order to ascertain whether the activation loop was observable in $SrcCD_{K295M}$ and **p**$SrcCD_{K295M}$ spectra, we employed amino-acid selective $^{13}C,^{15}N$ labeling as suggested by Langer (Langer et al. 2004) using growth media containing $^{13}C'$-labeled glycine and ^{15}N-labeled alanine. There is only a single GA entity in $SrcCD_{K295M}$. This lies in the activation loop

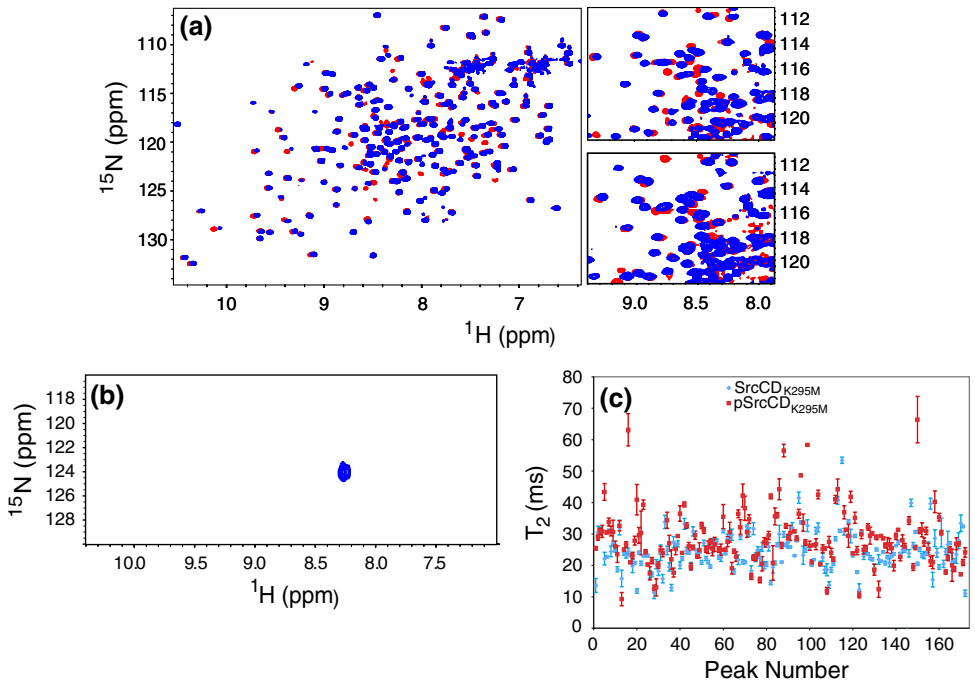

Fig. 1 **a** ^{15}N,^{1}H correlation (HSQC) of pSrcCD$_{K295M}$ (*red*) and SrcCD$_{K295M}$ (*blue*), data collected at 900 MHz. The *right panels* show expansions of the regions with significant changes. **b** For a selectively ^{13}C′-glycine,^{15}N-alanine labeled SrcCD$_{K295M}$, a single peak corresponding to the activation-segment conserved GA motif is seen in the ^{15}N,^{1}H plane of a 2D-HNCO experiment. **c** T_2 values at 800 MHz for partially-deuterated, uniformly ^{15}N-labeled SrcCD$_{K295M}$ (*blue*) and **p**SrcCD$_{K295M}$ (*red*)

sequence (^{416}YTARQ(**^{13}C′**)**G**(**^{15}N**)**A**^{422}). Notably, the GA motif is highly conserved in the SFKs. A 2D-HNCO spectrum produced a single peak, as expected, suggesting that at least a part of the activation loop was indeed observable by NMR (Fig. 1b). In order to test our ability to collect spin-relaxation data with high precision in the c-Src CD, we collected a compete set of backbone ^{15}N-relaxation data using TROSY-based relaxation experiments (Zhu et al. 2000). The representative spin–spin relaxation data (T_2) for SrcCD$_{K295M}$ and **p**SrcCD$_{K295M}$ reveal that the dynamic heterogeneity increases significantly upon phosphorylation (Fig. 1c).

In addition to characterizing the structural and dynamic changes that accompany SFK activation, NMR has proven to be an excellent method for drug screening (Hajduk et al. 1999; Vogtherr and Fiebig 2003) and has the potential to provide potentially valuable leads for the design of high-affinity inhibitors for the SFKs, including allosteric inhibitors that target sites remote from the ATP-binding pocket. Such inhibitors have been identified in other classes of protein kinases e. g., the ERK/MAP kinases (Chen et al. 2006). Our initial spectra display that structural changes upon binding to different small molecule inhibitors for both SrcCD$_{K295M}$ and **p**SrcCD$_{K295M}$ are indeed detectable by NMR.

The protocol described above applied on full-length c-Src (K295M mutant) and on the wild-type c-Src kinase domain (248–533, SrcCD$_{WT}$) produced lower yields (40–45% and ~30% of that obtained for SrcCD$_{K295}$ per liter of culture, respectively) but still sufficient material to generate nearly 1–3 NMR samples per liter of culture. In the latter case the lysis buffer was supplemented with an excess of the high-affinity c-Src inhibitor AP23464) (Dalgarno et al. 2006). We found that the addition of the inhibitor was crucial to allow binding of the fusion protein containing the wild-type construct to the cobalt beads during affinity purification. Both products gave excellent quality NMR spectra as displayed in Fig. 2.

As mentioned earlier, an important step in the NMR studies of c-Src is completion of resonance assignment. Given the size of the CD (~30 KDa) of c-Src, this procedure would be greatly facilitated by the partial or complete deuteration allowing and use of TROSY-based triple resonance experiments (Salzmann et al. 1998; Pervushin 2000) and the resultant increase in sensitivity at high magnetic fields. In our initial attempts to prepare uniformly ^{2}H-labeled protein, we found that protein expression was significantly decreased when the bacteria were grown in pure D_2O. However, we were able to successfully produce ^{2}H,^{15}N,^{13}C SrcCD$_{K295M}$ by supplementing M9 media prepared in H_2O with isotopically enriched algal lysates (Spectra-9 from Spectra Stable Isotopes) (Fiaux et al. 2004; Lohr et al. 2003). The amount of triple labeled protein obtained by using this procedure depended on the amount of algal lysate used in the growth medium. For example, by using 1.5 g/L of labeled material we got roughly 1/3 of the pure protein obtained from expression in standard M9 growth in H_2O (as described above). While this procedure does not result in a fully-deuterated protein, we believe that the sensitivity afforded by fractional deuteration should allow near complete resonance assignment as demonstrated in systems of similar size.

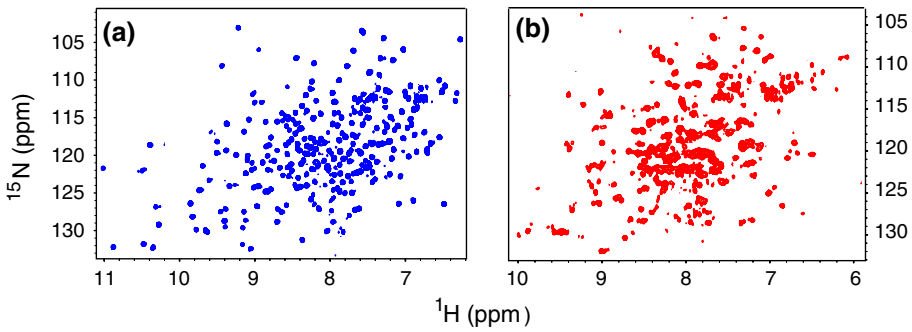

Fig. 2 **a** ^{1}H,^{15}N HSQC spectrum of fully-protonated $SrcCD_{WT}$ complexed with AP23464 and **b** of fully-protonated full-length c-Src. Spectra were acquired at 800 MHz

We have optimized a protocol for producing the c-Src kinase domain both in the unphosphorylated form and that selectively phosphorylated on the activating tyrosine residue (Y_{act}) in sufficient quantities to make solution NMR studies feasible. All the constructs generated in the relevant states of phosphorylation produce NMR spectra of excellent quality indicating that complete or near-complete resonance assignments should indeed be possible on this pharmacologically significant signaling molecule. We are currently applying this protocol to other members of the SFKs.

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