Both proline-rich sequences in the TH region of Bruton's tyrosine kinase stabilize intermolecular interactions with the SH3 domain

Henrik Hansson[a], C.I. Edvard Smith[b,c], Torleif Härd[a,*]

[a] *Department of Biotechnology, Royal Institute of Technology (KTH), SCFAB, S-106 91 Stockholm, Sweden*
[b] *Department of Biosciences at Novum, Karolinska Institute, S-141 57 Huddinge, Sweden*
[c] *Clinical Research Center, Karolinska Institute, Huddinge University Hospital, S-141 86 Huddinge, Sweden*

Received 20 August 2001; revised 5 October 2001; accepted 5 October 2001

First published online 22 October 2001

Edited by Guilio Superti-Furga

Abstract The Tec homology (TH) region located N-terminal to the Src homology 3 (SH3) domain of Bruton's tyrosine kinase (Btk) contains two proline-rich SH3-binding sequences (PRRs). We have previously demonstrated that the TH region acts to stabilize intermolecular interactions in N-terminally extended SH3 (PRR–SH3) fragments. Here, we analyze six PRR–SH3 fragments with different proline-to-alanine substitutions in the two PRRs. Gel permeation chromatography and nuclear magnetic resonance spectroscopy show that both PRRs can stabilize self-association. This observation provides an explanation to why the TH region of Btk makes intermolecular interactions, whereas the corresponding interaction in the related Itk kinase with only one PRR, is intramolecular.

Key words: Bruton's tyrosine kinase; Src homology 3; Dimerization; Nuclear magnetic resonance; Gel permeation chromatography; Signal transduction

1. Introduction

Bruton's tyrosine kinase (Btk) is a multi-domain enzyme with a key role in B-lymphocyte development. Mutations in the gene encoding the kinase cause the hereditary immunodeficiency X-linked agammaglobulinemia [1–3]. Sequence homology and subdomain composition place Btk in the Tec-family of non-receptor kinases, which also includes the Itk, Tec, Bmx, and Txk kinases [4]. Btk is activated by Src-family kinases through a tyrosine phosphorylation within the kinase domain followed by autophosphorylation of Y223 in the Src homology 3 (SH3) domain [5–7]. Phosphorylation of Y223 presumably disrupts interactions between SH3 and an SH3-binding peptide, and this disruption may then lead to activation.

The Tec homology (TH) region of Btk, located immediately N-terminal to the SH3 domain, contains two proline-rich regions (PRR) with SH3-binding PxxP motifs, whereas the related Itk kinase contains only one such region. Studies of Itk suggest that the PRR of the TH region can fold onto and bind the Itk SH3 in an intramolecular association [8]. This is similar to what is observed in the Src-family kinases c-Src and Hck as well as for the nuclear protein tyrosine kinase c-Abl, where the inactive enzymes are stabilized by intramolecular interactions involving SH3 domains [9–11].

We and others have previously determined the structure of the Btk SH3 domain [12,13]. We have also studied an N-terminally extended fragment of Btk SH3 that contains the proline-rich sequences of the TH region, the PRR–SH3 fragment (Fig. 1). In contrast to the situation in Itk, we found that the Btk SH3 domain interacts with the TH region in an intermolecular manner and that experimental data are consistent with dimerization of PRR–SH3 fragments with a K_d of approximately 60 μM [14]. Here, we address the question of the relative role of the two different PRR sequences in this interaction. Various PRR–SH3 fragments are produced in which either two or all four of the proline residues in one or both PRRs are replaced by alanines. We then characterize these mutant fragments with gel permeation chromatography (GPC) to assess the state of self-association, and by nuclear magnetic resonance (NMR) spectroscopy to examine the extent of PRR binding to the SH3 domain. We find that both PRRs can act to stabilize intermolecular association and that only the destruction of all PxxP-motifs in the two PRRs significantly shifts the equilibrium towards a monomeric state of PRR–SH3. The results provide a basis for a discussion of the structural properties of the interactions and of the differences between Itk and Btk.

2. Materials and methods

2.1. Site-directed mutagenesis

The expression plasmid encoding residues 178–275 (the PRR–SH3 fragment) of human Btk as a glutathione *S*-transferase (GST) fusion has been described previously [14]. Mutations in this plasmid were performed using QuickChange™ (Stratagene) following instructions provided by the manufacturer. Six altered gene fragments were generated in four steps using eight different PCR primers (Cybergene AB, Sweden). In each step, two proline codons were replaced with alanine codons by single base-pair substitutions (CCC to GCC, CCT to GCT, CCA to GCA, or CCG to GCG). The amino acid sequences of the wild type and mutated protein fragments and the notations of the different fragments are shown in Fig. 1B. The mutated plasmid vectors were purified using Qiagen SpinPrep™ and the DNA sequence was confirmed. In one case, PRR^{N4}, an additional single base-pair substitution had occurred causing an incidental single amino acid residue substitution: E193V.

*Corresponding author. Fax: (46)-8-5537 8358.
E-mail address: torleif.hard@biotech.kth.se (T. Härd).

Abbreviations: Btk, Bruton's tyrosine kinase; SH3 domain, Src homology 3 domain; TH region, Tec homology region; PRR–SH3, N-terminally extended Btk SH3 domain containing the proline-rich sequences of the TH region; NMR, nuclear magnetic resonance; GPC, gel permeation chromatography; GST, glutathione *S*-transferase

0014-5793/01/$20.00
PII: S0014-5793(01)03018-6

2.2. Gene expression and protein purification

The production of unlabeled and ^{15}N-labeled Btk PRR–SH3 and mutated variants of PRR–SH3 was carried out essentially as reported previously [12,14], with the following exceptions: The bacterial strain *Escherichia coli* BL21 (DE3) codon+RP (Stratagene) was used. Ion exchange was not used for purification. Instead, the protein that could be cleaved off from GSH–Sepharose was concentrated in a 50 ml Amicon stir cell (YM3) and heat-treated at 65°C for 15 min followed by immediate centrifugation at 24 460×*g* and 4°C. This procedure removed most of the contaminant GST protein. Gel filtration was then performed in 40 mM ammonium acetate buffer at pH 6.3 and the eluted protein was lyophilized and dissolved in experimental buffer (20 mM sodium phosphate buffer at pH 6.5 containing 20 mM NaCl and 1 mM EDTA). The pH was set to 6.5.

2.3. GPC

Analytical GPC was performed as described earlier [14], using the same Superdex 75 HR 10/30 column (Amersham Pharmacia Biotech) which has a void volume (V_M) of 7.5 ml and a totally included solute volume (V_S) of 10.8 ml. The elution of protein was monitored by measuring absorbance at 280 nm over 10 mm optical pathlength. The elution volumes (V_e) of globular protein standards were recorded to demonstrate a linear relationship between V_e and the logarithm of the molecular weight in the elution volume interval 8.6–13.6 ml. The GPC band shapes of the standards were symmetric, as expected. The V_e values of wild type PRR–SH3 and the standards in the present set of experiments were also comparable, within 0.1–0.2 ml, to those of the previously described experiments [14].

2.4. NMR spectroscopy

NMR was measured at 30°C on a Bruker Avance™ spectrometer operating at 500 MHz ^{1}H frequency using a 5 mm triple-resonance (^{1}H/^{15}N/^{13}C) probe. Two-dimensional ^{1}H/^{15}N HSQC experiments were recorded and processed using conventional methods [15], and analyzed using Ansig for Windows [16].

3. Results

3.1. Mutated PRR–SH3 fragments

The Btk contains two PRRs with SH3-binding properties located immediately N-terminal to the SH3 domain. The sequences are almost identical – KKPLPP(T/E)P – where the most N-terminal PRR^N contains a Thr residue and the following PRR^C contains a Glu residue. Hence, both regions contain *two* PxxP motifs, which is an essential signature of SH3-binding peptides. To elucidate the role of the two PRR motifs and also, to some extent, the relative importance of the different PxxP motifs within the PRRs, we constructed six mutant protein fragments (Fig. 1) with two, four, or eight proline-to-alanine replacements. In three fragments, we changed the second of the PxxP sequences to AxxA in the first (PRR^{N2}) or second (PRR^{C2}) PRR, and in both regions (PRR^{N2C2}). In the other three fragments, we changed all prolines in the first (PRR^{N4}), second (PRR^{C4}), and both PRRs (PRR^{N4C4}) to alanines. All mutated PRR–SH3 fragments were soluble and could be purified to homogeneity.

3.2. GPC

The different intermolecular association properties of the wild type PRR–SH3 and mutant fragments can be monitored by the elution volume and band shapes during GPC, and by the concentration dependence of these properties [14]. GPC chromatograms of wild type PRR–SH3 and the PRR mutants are shown in Fig. 2, where panels A–C show results for loading approximately equal 1 mM protein samples and panel D contains a comparison of the concentration dependencies of wild type PRR–SH3 and the PRR^{N4C4} mutant. When loaded at 1.0 mM concentration, the wild type PRR–SH3 elutes at 11.0 ml with a tailing band shape, which is indicative of self-association.

Fig. 2A and B show that mutations in both PRR^N and in PRR^C shift the elution to larger V_e values, suggesting smaller average molecular weights during GPC. However, the band shapes are still asymmetric, indicating self-association. Mutations in PRR^N results in a larger V_e shift than mutations in PRR^C. In both PRRs, mutations of two prolines shift the elution, but mutations of all four prolines result in even larger shifts (the effect of reducing nominal molecular weights by replacing Pro with smaller Ala sidechains can be neglected).

The effect of simultaneous mutations in the two PRRs is illustrated in Fig. 2C. Again, the effect on V_e of mutating all four prolines in the PRRs is larger than when only two replacements are made in each PRR. In addition, the elution band shape of the eight-mutation PRR^{N4C4} fragment is obviously more symmetric than that of the wild type PRR–SH3. The differences are also apparent when comparing the con-

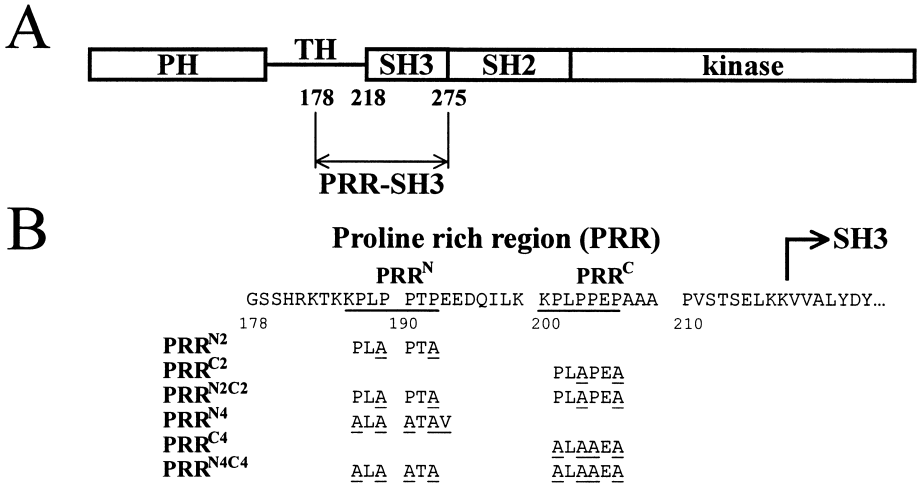

Fig. 1. A: Overview of Btk containing the pleckstrin homology (PH) domain, TH region, SH3 and SH2 domains and the catalytic domain (kinase). The numbers correspond to the N-terminal and C-terminal residues of the PRR–SH3 fragment and to the first structured residue of the SH3 domain. B: The sequence of the PRR part of Btk together with an overview of the amino acid residue substitutions that are made in the various PRR–SH3 mutants.

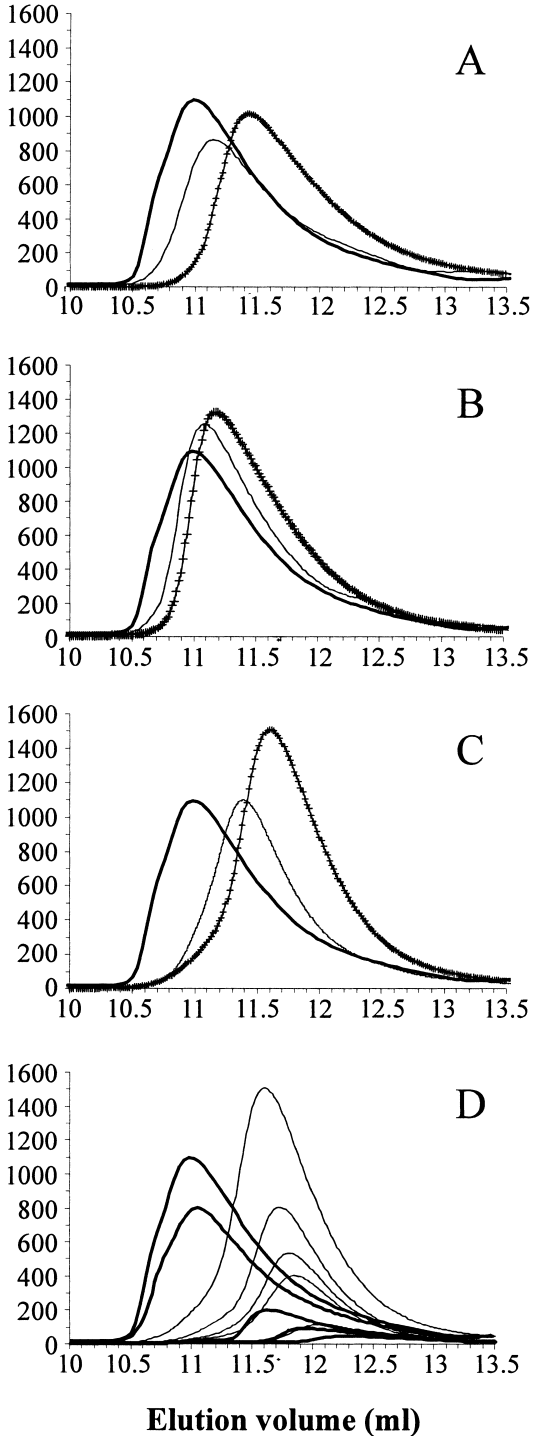

Fig. 2. Effects of amino acid residue substitutions (Pro to Ala) on GPC elution profiles. The elution of wild type PRR–SH3 at 1.0 mM loaded concentration is shown with a thick line in all four panels. A: Substitutions in PRR^{N}; thin line: PRR^{N2} (loaded concentration of 0.95 mM), bar crossed line: PRR^{N4} (1.1 mM). B: Substitutions in PRR^{C}; thin line: PRR^{C2} (0.88 mM), bar crossed line: PRR^{C4} (1.0 mM). C: Substitutions in both PRR^{N} and PRR^{C}; thin line: PRR^{N2C2} (1.0 mM), bar crossed line: PRR^{N4C4} (1.0 mM). D: Comparison of GPC elution profiles with different concentrations of loaded protein samples. Thick lines: wild type PRR–SH3 (1.0, 0.8, 0.2, 0.1 and 0.05 mM; [14]). Thin lines: PRR^{N4C4} (1.0, 0.5, 0.27, 0.23 and 0.07 mM). The scale at the abscissa indicates absorbance at 280 nm in mAU.

centration dependencies of the two proteins. As can be seen in Fig. 2D, the elution behavior of PRR^{N4C4} is almost independent of loaded concentration in the range 0.07–0.99 mM. The shift in V_e towards larger values, the symmetric band shape, and the small concentration dependence of PRR^{N4C4} suggest that the self-association equilibrium of this fragment is shifted towards monomeric behavior, as compared to the behavior of wild type PRR–SH3.

3.3. NMR spectroscopy

The self-association or dimerization equilibrium can also be monitored by the NMR chemical shifts of certain residues close to the peptide-binding pocket on the SH3 domain [14]. The backbone amide chemical shifts of three mutant fragments (PRR^{N4}, PRR^{C4} and PRR^{N4C4}) were assigned and compared to the wild type PRR–SH3 as well as to the isolated SH3 domain. In Fig. 3A, it can be seen that mutations in either PRR^{N} or PRR^{C} reduce the difference between NMR spectra of the SH3 domain and the N-terminally extended fragment PRR–SH3, but none of the fragments display an SH3 spectrum that is identical to that of the isolated domain. However, when both PRRs are mutated, the chemical shift differences between PRR^{N4C4} and the isolated SH3 domain are very small (Fig. 3B). In other words, the NMR spectrum of the isolated Btk SH3 domain is very similar to that of the SH3 domain in PRR^{N4C4}. The effect of the self-association on the NMR spectrum of SH3 that occurs in N-terminally extended fragments [14] is therefore reversed by mutations in the two PRRs, as illustrated in Fig. 4. These results are consistent with the GPC data and demonstrate that no, or only very weak, interactions exist between the SH3 domain and the TH region (at the 1 mM concentration level) if all eight prolines in the PRRs are mutated, whereas mutation of only one of the two PRRs results in an intermediate behavior.

4. Discussion

Self-regulation through intramolecular interactions has been inferred for some non-receptor kinases in the Src family [9,10]. However, the picture is not clear regarding the regulation of Tec-family kinases. For the SH3 domain of Itk, it was shown that an SH3-binding sequence in an N-terminally extended Itk SH3 fragment binds to SH3 in an intramolecular manner [8]. On the other hand, it has recently been shown that the SH2 and SH3 domains of Itk can form intermolecular complexes [17].

We previously demonstrated that N-terminally extended fragments of the Btk SH3 domain that include the proline-rich sequences of the adjacent TH region undergo self-association, most likely in the form of dimerization [14]. Hence, there may be mechanistic differences in the self-regulation of Btk and Itk. At the sequence level, Btk contains two PRRs N-terminal to the SH3 domain, whereas Itk contains only one. The sequence of the Itk PRR is identical to Btk PRR^{N}, but it is located at a distance (in sequence) from the SH3 domain of Itk, similar to that of PRR^{C} in Btk.

The objective of the present work was to examine the relative role of the two PRR sequences in the intermolecular interaction observed with Btk PRR–SH3 fragments. For this purpose, we produced six protein fragments with different mutations in the PRR sequences. The association state of these fragments was monitored by GPC and the extent of

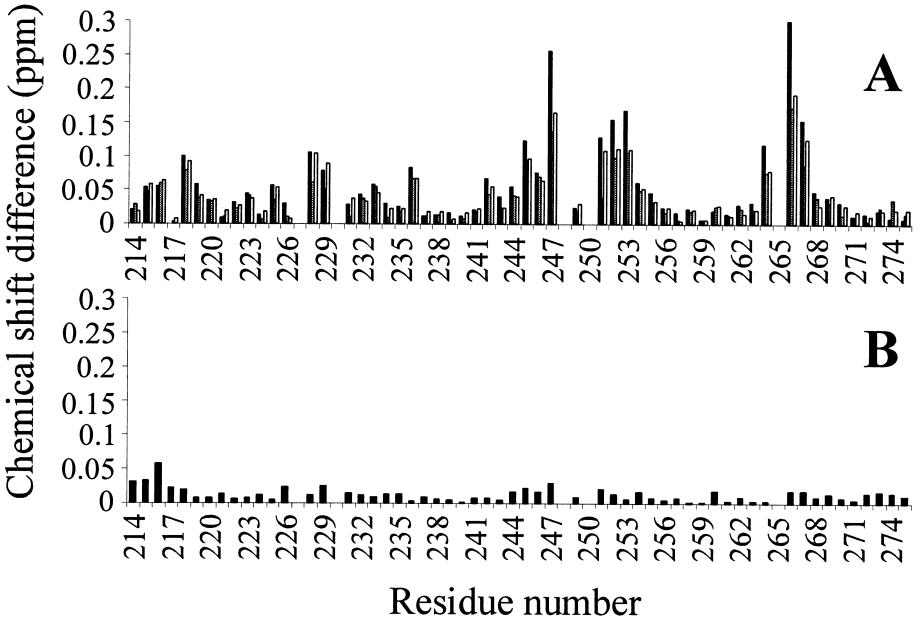

Fig. 3. Comparison of backbone amide chemical shifts within the Btk SH3 domain. A: Wild type PRR–SH3 (solid), PRR^{N4} (hatched) and PRR^{C4} (open) compared to isolated SH3 domain without N-terminal extension (Btk SH3). B: PRR^{N4C4} compared to Btk SH3. Backbone amide ^{1}H and ^{15}N chemical shifts were extracted from ^{15}N HSQC spectra of protein samples at similar 1 mM concentrations, and total chemical shift differences were calculated as $([0.15\times\Delta(\delta_{N^H})]^2 + [\Delta(\delta_{H^N})]^2)^{1/2}$.

interactions present at the peptide–SH3 binding interface was monitored by observing changes in NMR chemical shifts.

Both GPC and NMR data indicate that the intermolecular interaction is more dependent on PRR^N than on PRR^C. However, the differences are small and the interactions at the peptide-binding pocket on SH3 can only be completely disrupted if both PRR^N and PRR^C are mutated (Figs. 3 and 4). In concert, the GPC data show that mutation of both PRRs shifts the association equilibrium more towards the monomeric state than mutations in only one of the PRRs (Fig. 2). Hence, we conclude that both PRR sequences in Btk can interact with the SH3 domain in an intermolecular manner. It may appear superfluous for a protein to evolve this redundancy for intermolecular interactions, but the fact that both PRRs contribute to the stabilization of these suggests that they are both required for function in vivo. A possible explanation for the sequence differences between Itk and Btk is therefore that the, presumably intramolecular, self-regulation of Itk does not require more than one PRR. To our knowledge there are no apparent functional differences that could be directly related to the single versus double PRRs in Itk and Btk, respectively. However, the putative partners of these kinases differ, making direct comparisons difficult [4]. Since the PRR differences are conserved over species barriers, a conserved functional importance is suggested. Moreover, functionally relevant sequence variations are frequently seen among members in other gene families, such as Src family kinases [18]. It should also be pointed out that the interactions seen in the PRR–SH3 constructs may differ from those found in intact full-length kinases.

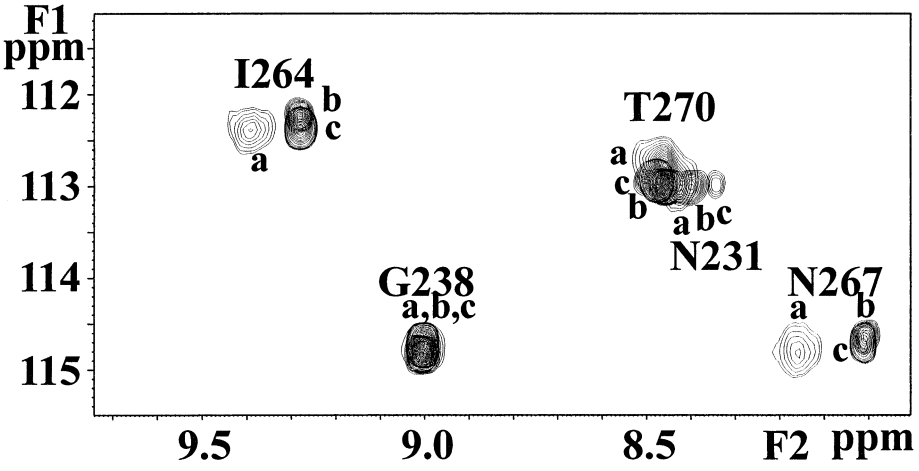

Fig. 4. Section of the ^{15}N HSQC spectrum of wild type PRR–SH3 (a), Btk SH3 without N-terminal extension (b) and PRR^{N4C4} (c). The chemical shifts of the I264 and N267 resonances in wild type PRR–SH3 have previously been shown to be concentration-dependent due to self-association (see [14]).

The PRR sequences in the TH region have been assigned to the class I type in which the basic residue binding in the specificity pocket of SH3 is located N-terminal to the PxxP motifs [8]. The predicted critical prolines that are expected to make van der Waals contacts in the binding pocket are those at positions P_0 and P_3 positions (notation as in [19]), i.e. Pro189 and Pro192 in PRR^N, and Pro203 and Pro206 in PRR^C. However, our data show that mutation at these four positions is not sufficient to break all intermolecular interactions. It is possible that the structure at the peptide–SH3 interface remains essentially unchanged and that interactions remaining after mutation at P_0 and P_3 are sufficient to preserve a complex. A second possibility is that the PRR interactions shift in register when one of the PxxP motifs is mutated and that their SH3-interacting role is taken over by the prolines originally present at the P_{-2} and P_1 positions. Such a shift would bring the basic lysine residue out-of-register with the specificity pocket. However, we note that there are *two* adjacent lysine residues at the N-terminal of both PRRs and that PRR^N contains additional basic residues (Arg182 and K183) further towards the N-terminal. These may then be available to interact with the SH3 domain in a 'shifted' PRR–SH3 interaction. This second explanation would imply that there are possibilities for alternative SH3-binding modes, not only between the two PRR sequences, but also within each PRR.

Finally, there are two points to note with regard to the possible structure of a dimeric PRR–SH3 fragment. First, it cannot be excluded that there are other intermolecular interactions than those mediated by the PRRs. The evidence for this is some remaining concentration dependence in the GPC elution volume of the PRR^{N4C4} mutant (Fig. 2D). It is unlikely that this is due to binding of mutated PRR sequences to SH3, because the NMR spectrum of the PRR-binding site on SH3 is very similar to that of the isolated SH3 domain. Hence, other sites in the N-terminal or in the SH3 domain might contribute to intermolecular interactions. Second, the rather small differences between the contributions from PRR^N and PRR^C to self-association equilibrium imply that the free energy differences between the different binding modes are small as well. Hence, several conformational states might co-exist at equilibrium. There are three possible states for a dimer where both N-terminals bind across the dimer interface. Additional states may exist in which only one of the N-terminals binds the other monomer and the other is uncomplexed in solution. The presence of several co-existing states is not directly supported by the NMR spectrum of PRR–SH3, which appears to consist of only a single set of resonances. This fact may, on the other hand, be due to averaging caused by rapid interconversion between conformational states. We note that the possible presence of several different states in equilibrium has rather severe implications for a solution structure determination of the PRR–SH3 fragment of Btk.

Acknowledgements: This work was supported by the Swedish Foundation for Strategic Research (SSF), the Knut and Alice Wallenberg Foundation and the Swedish Cancer Fund.

References

[1] Vetrie, D., Vorechovsky, I., Sideras, P., Holland, J., Davies, A., Flinter, F., Hammarström, L., Kinnon, C., Levinsky, R., Bobrow, M., Smith, C.I.E. and Bentley, D.R. (1993) Nature 361, 226–233.
[2] Tsukada, S., Saffran, D.C., Rawlings, D.J., Parolini, O., Allen, R.C., Klisak, I., Sparkes, R.S., Kubagawa, H., Mohandas, T., Quan, S., Belmont, J.W., Cooper, M.D., Conley, M.E. and Witte, O.N. (1993) Cell 72, 279–290.
[3] Vihinen, M., Arredondo-Vega, F.X., Casanova, J.L., Etzioni, A., Giliani, S., Hammarström, L., Hershfield, M.S., Heyworth, P.G., Hsu, A.P.A.L., Lappalainen, I., Notarangelo, L.D., Puck, J.M., Reith, W., Roos, D., Schumacher, R.F., Schwarz, K., Vezzoni, P., Villa, A., Valiaho, J. and Smith, C.I.E. (2001) Adv. Genet. 43, 103–188.
[4] Smith, C.I.E., Islam, T.C., Mattsson, P.T., Mohamed, A.J., Nore, B.F. and Vihinen, M. (2001) Bioessays 23, 436–446.
[5] Rawlings, D.J., Scharenberg, A.M., Park, H., Wahl, M.I., Lin, S., Kato, R.M., Fluckiger, A.-C., Witte, O.N. and Kinet, J.-P. (1996) Science 271, 822–825.
[6] Mahajan, S., Fargnoli, J., Burkhardt, A.L., Kut, S.A., Saouaf, S.J. and Bolen, J.B. (1995) Mol. Cell. Biol. 15, 5304–5311.
[7] Park, H., Wahl, M.I., Afar, D.E.H., Turck, C.W., Rawlings, D.J., Tam, C., Scharenberg, A.M., Kinet, J.-P. and Witte, O.N. (1996) Immunity 4, 515–525.
[8] Andreotti, A.H., Bunnell, S.C., Feng, S., Berg, L.J. and Schreiber, S.L. (1997) Nature 385, 93–97.
[9] Sicheri, F., Moarefi, I. and Kuriyan, J. (1997) Nature 385, 602–609.
[10] Xu, W., Harrison, S.C. and Eck, M.J. (1997) Nature 385, 595–602.
[11] Barilá, D. and Superti-Furga, G. (1998) Nat. Genet. 18, 280–282.
[12] Hansson, H., Mattsson, P.T., Allard, P., Haapaniemi, P., Vihinen, M., Smith, C.I.E. and Härd, T. (1998) Biochemistry 37, 2912–2924.
[13] Tzeng, S.R., Lou, Y.C., Pai, M.T., Jain, M.L. and Cheng, J.W. (2000) J. Biomol. NMR 16, 303–312.
[14] Hansson, H., Okoh, M., Smith, C.I.E., Vihinen, M. and Härd, T. (2001) FEBS Lett. 489, 67–70.
[15] Cavanagh, J., Fairbrother, W.J., Palmer, A.G. and Skelton, N.J. (1996) Protein NMR Spectroscopy Principles and Practice, Academic Press, London.
[16] Helgstand, M., Kraulis, P., Allard, P. and Härd, T. (2000) J. Biomol. NMR 18, 329–336.
[17] Brazin, K.N., Fulton, D.B. and Andreotti, A.H. (2000) J. Mol. Biol. 302, 607–623.
[18] Resh, M.D. (1994) Cell 76, 411–413.
[19] Yu, H., Chen, J.K., Feng, S., Dalgarno, D.C., Brauer, A.W. and Schreiber, S.L. (1994) Cell 76, 933–945.