NMR characterizations of an amyloidogenic conformational ensemble of the PI3K SH3 domain

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Abstract

Amyloid formation is associated with structural changes of native polypeptides to monomeric intermediate states and their self-assembly into insoluble aggregates. Characterizations of the amyloidogenic intermediate state are, therefore, of great importance in understanding the early stage of amyloidogenesis. Here, we present NMR investigations of the structural and dynamic properties of the acid-unfolded amyloidogenic intermediate state of the phosphatidylinositol 3-kinase (PI3K) SH3 domain—a model peptide. The monomeric amyloidogenic state of the SH3 domain studied at pH 2.0 (35°C) was shown to be substantially disordered with no secondary structural preferences. ^{15}N NMR relaxation experiments indicated that the unfolded polypeptide is highly flexible on a subnanosecond timescale when observed under the amyloidogenic condition (pH 2.0, 35°C). However, more restricted motions were detected in residues located primarily in the β-strands as well as in a loop in the native fold. In addition, nonnative long-range interactions were observed between the residues with the reduced flexibility by paramagnetic relaxation enhancement (PRE) experiments. These indicate that the acid-unfolded state of the SH3 domain adopts a partly folded conformation through nonnative long-range contacts between the dynamically restricted residues at the amyloid-forming condition.

Keywords: amyloids; PI3K SH3; NMR; dynamics; amyloidogenic intermediate; long-range interactions; PRE

Interest in the unfolded and partly folded states of proteins has been growing because of their important roles in a variety of biological processes, including cellular signaling and transcriptional activation (Shortle 1993; Dyson and Wright 2005). In addition, it is widely accepted that partly structured states of proteins are involved in early events in the formation of amyloid, which underlies a number of debilitating human diseases such as Alzheimer's and Parkinson's diseases (Dobson 2003). Thus, characterization of the unfolded and partially folded states of proteins is of central importance in understanding the molecular mechanism of the biological processes as well as the amyloid diseases. Multinuclear, multidimensional NMR spectroscopy has proven to be a powerful technique to investigate site-specific structural and dynamic properties of nonnative states of proteins (Shortle 1996; Dyson and Wright 2004; Redfield 2004). Extensive NMR studies of denatured states of various nonamyloidogenic proteins have indicated the presence of

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Abbreviations: NMR, nuclear magnetic resonance; HSQC, heteronuclear single-quantum coherence; PI3K SH3, phosphatidylinositol 3-kinase Src homology 3; R_2, transverse relaxation rate; NOE, nuclear Overhauser effect; $R_{1\rho}$, relaxation rate in the rotating frame; H/D, hydrogen/deuterium; PRE, paramagnetic relaxation enhancement; MTSL, (1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl) methanethiosulfonate; UV, ultraviolet; CD, circular dichroism.

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a native-like topology and/or hydrophobic clusters under denaturing conditions (Eliezer et al. 2000; Katou et al. 2001; Shortle and Ackerman 2001; Klein-Seetharaman et al. 2002; Lietzow et al. 2002). To gain insight into mechanisms of protein folding and misfolding, it is important to compare the conformational ensemble of an amyloidogenic protein with other nonamyloidogenic denatured states.

Phosphatidylinositol 3-kinase (PI3K) SH3 domain is an 85-residue, β-structured, protein interaction module (Fig. 1) that recognizes proline-rich ligands (Pawson 1995; Liang et al. 1996). Recently, the small protein was shown to form amyloid fibrils at pH 2.0 (Guijarro et al. 1998). The non-disease related SH3 domain has served as an excellent model system for studies of the structural properties of amyloid fibrils and the molecular mechanism of amyloid formation (Jimenez et al. 1999; Zurdo et al. 2001a,b; de Laureto et al. 2003; Carulla et al. 2005). Here, we report the structural and dynamic properties of the acid-unfolded SH3 domain at the amyloidogenic condition of pH 2.0 and 35°C. We employed relaxation NMR and chemical shift analyses for the studies of amyloidogenic states, which showed that the SH3 domain was largely unfolded with no secondary structural preferences at the amyloid-forming condition. Relatively less flexible regions in subnanosecond timescales were, however, identified. Paramagnetic relaxation enhancement experiments also demonstrated that long-range contacts between the residues with restricted segmental motions persist in the acid-unfolded state at pH 2.0. These suggest that the amyloidogenic intermediate states adopt partly folded conformations, supporting the hypothesis that partly folded amyloidogenic intermediates play a critical role in the amyloid formations. The structural and dynamic properties of the acid-unfolded amyloidogenic states were compared with other acid-unfolded, nonamyloidogenic states of proteins.

Results

Secondary structure

A ^{1}H-^{15}N heteronuclear single-quantum coherence (HSQC) NMR spectrum of the SH3 domain (0.1 mM) was obtained under the amyloid-forming condition of pH 2.0 and 35°C (Fig. 2). Minimal dispersion observed in the ^{1}H dimension indicated that the native structure of the SH3 domain is disrupted under these conditions (Dyson and Wright 2004). The NMR resonances, however, were sufficiently separated in the ^{15}N dimension to permit complete backbone assignments by conventional 3D NMR experiments. Thus the $^{13}C^{\alpha}$, $^{13}C^{\beta}$, $^{13}C'$, and $^{1}H^{\alpha}$ chemical shifts could be analyzed to investigate the secondary structure of the conformational ensemble in the acid-unfolded amyloidogenic state of the SH3 domain (Fig. 3). Chemical shift analyses using the software PECAN (Eghbalnia et al. 2005) showed that none of the chemical shifts deviated significantly from those for a random coil conformation. This suggests that in the amyloidogenic state, the SH3 domain adopts a highly flexible conformational ensemble with no secondary structural preferences at 35°C.

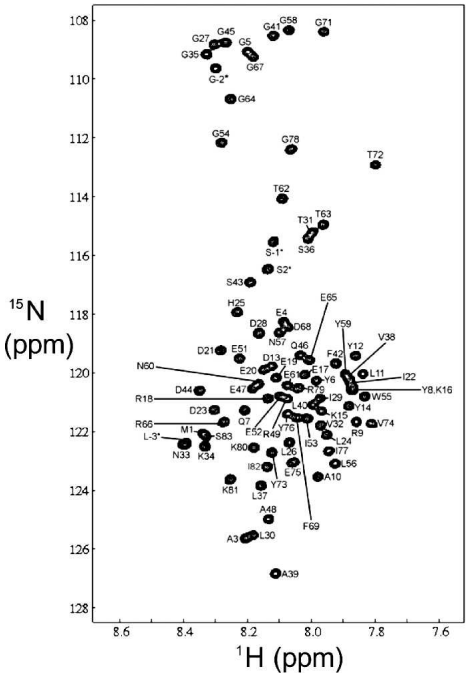

Figure 2. The ^{1}H-^{15}N HSQC NMR spectrum of the PI3K SH3 domain at 35°C (pH 2.0). Signals from the additional five N-terminal residues resulting from the expression construct are identified by asterisks.

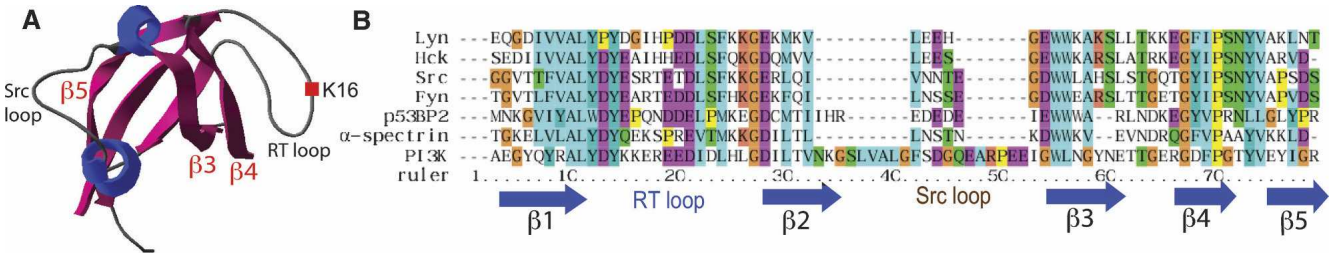

Figure 1. (*A*) Crystal structure of the PI3K SH3 domain drawn from the PDB code (1PHT) (Liang et al. 1996). (*B*) Sequence alignment of SH3 domains.

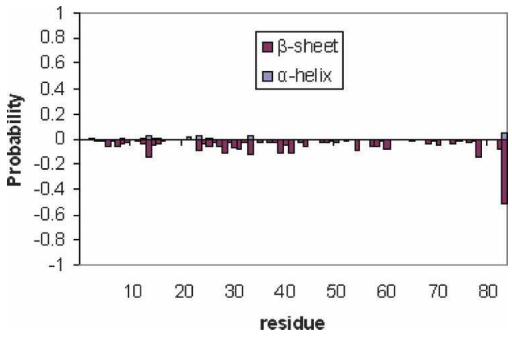

Figure 3. Secondary structural preferences of the SH3 domain at pH 2.0 based on the assigned $^{13}C^{\alpha}$, $^{13}C^{\beta}$, $^{13}C'$, and $^{1}H^{\alpha}$ chemical shifts. The probability was calculated by using the software PECAN (Eghbalnia et al. 2005). The additional five residues at the N terminus were not included in the analysis.

Backbone dynamics

^{15}N relaxation rates (R_2) were measured at a proton frequency of 500 MHz (Fig. 4A) to investigate dynamic properties of the amyloidogenic, acid-unfolded state (pH 2.0 and 35°C). The overall slow relaxation rates indicate that the SH3 domain is highly flexible under these conditions. The relaxation rates observed for residues 8–25, 51–63, and 72–78, were, however, notably faster than others, suggesting that those fragments may undergo restricted motion and/or conformational exchange on the millisecond timescale. Relaxation rates in the rotating-frame ($R_{1\rho}$), which are less sensitive to conformational fluctuations (Szyperski et al. 1993), determined under the same conditions (Fig. 4B), showed almost identical distributions, suggesting that the faster relaxation rates are due mainly to restricted motion of the polypeptide chain. The rigid motion of these residues is also evident in the heteronuclear NOE profiles obtained at proton frequencies of 500 and 800 MHz (Fig. 5). The ^{1}H-^{15}N NOE values at 500 MHz (Fig. 5A) were negative for almost all of the residues, whereas at 800 MHz, the values were almost all positive. This strongly indicates that the acidic amyloidogenic state fluctuates on a subnanosecond timescale.

Paramagnetic relaxation enhancement

The uneven distribution of the relaxation rate profiles has been interpreted as a presence of weak clusters stabilized by local contacts and/or long-range interactions in the denatured states (Schwalbe et al. 1997; Eliezer et al. 2000; Bussell and Eliezer 2001; Yao et al. 2001; Klein-Seetharaman et al. 2002; Ohnishi and Shortle 2003; Platt et al. 2005). Paramagnetic relaxation enhancement (PRE) experiments were, therefore, carried out on the cystein-containing mutant SH3 domain (K16C), which will allow us to probe long-range contacts in the amyloidogenic conformational ensemble. Lysine 16 with a relatively higher relaxation rate (Fig. 4) was chosen to investigate a local chain compactness of the N-terminal region as well as long-range interactions between the residues with restricted motions. The nitroxide spin label, MTSL, attached to the residue C16 will cause extensive line broadenings, and thus the NMR signals will be decreased for residues in the vicinity of the spin-labeled C16 (<15–20 Å). In a flexible random coil, NMR signals of ~15 residues from the site of spin labeling are predicted to be decreased in intensity by >50–60% (Lietzow et al. 2002). In the amyloidogenic state of the SH3 domain, the PRE effects on the nearby residues are far more pronounced than predicted for the random coil polypeptide (Fig. 6), suggesting a local chain compaction of the N-terminal region. In addition, distant residues (48–60, 67–78) are clearly affected by the spin label attached to residue C16, a strong indicator of the long-range contacts in the conformational ensemble (Fig. 6). Interestingly, residues with the reduced mobility detected by the relaxation experiments are mainly affected by the spin label (Figs. 4, 6), which indicates that the acid-unfolded state adopts partially folded conformations through long-range interactions between the dynamically restricted regions of the SH3 domain.

Discussion

Characterization of the structural and dynamic properties of amyloidogenic states is vital to understanding the molecular mechanism of amyloid formation. It is widely believed that

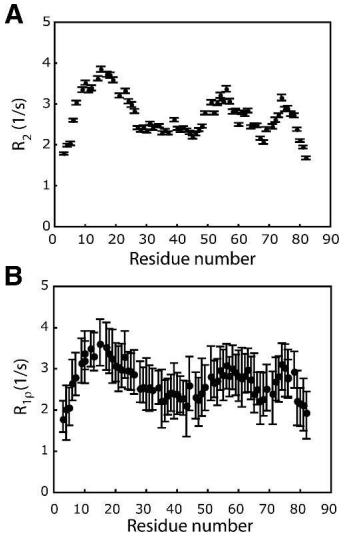

Figure 4. (*A*) Relaxation rates (R_2) of the SH3 domain obtained at the proton frequency of 500 MHz. (*B*) Relaxation rates in the rotating frame ($R_{1\rho}$). The $R_{1\rho}$ values were measured using the room temperature probe at the proton frequency of 500 MHz, and thus the larger errors probably originate from the lower signal-to-noise ratio compared to data in *A* obtained with the cold probe.

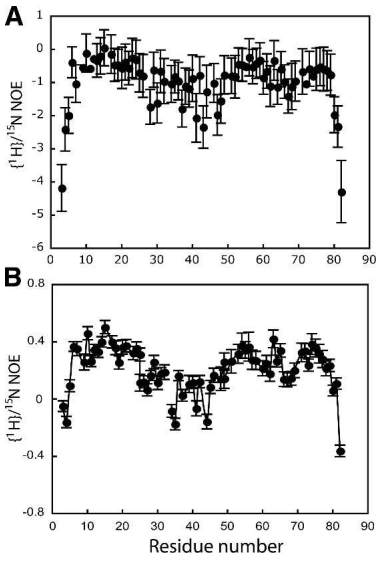

Figure 5. The ^{1}H-^{15}N heteronuclear NOE obtained at two different field strengths of 500 (*A*) and 800 (*B*) MHz. The larger errors in *A* measured with the room temperature probe originate from the lower signal-to-noise ratio compared to data in *B* obtained with the cold probe.

partly folded amyloid precursors are involved in amyloidogenesis. Residue-specific structural analyses by hydrogen/deuterium (H/D) exchange (Liu et al. 2000) and denaturant titration NMR experiments (McParland et al. 2002), respectively, have been reported for two β-structured amyloidogenic proteins, transthyretin (TTR) and β(2)-microglobulin. These NMR studies demonstrated that the monomeric amyloidogenic state adopts partly structured conformations under the amyloidogenic pH of 4–5. The β-barrel SH3 domain studied here has been shown to form amyloids at the lower pH of 2.0 (Guijarro et al. 1998). The chemical shift data reported here indicate that the SH3 domain does not possess secondary structural preferences under the acidic amyloidogenic condition (pH 2.0, 35°C). Recently, unstructured states of myoglobin probed by CD spectroscopy were also demonstrated to form amyloid fibrils at 65°C (Fandrich et al. 2003). These results suggest that secondary structure of the aggregation-prone state may not be critical for the effective amyloid formations. However, the acid-unfolded state of the SH3 domain was shown to adopt relatively compact conformations based on the hydrodynamic radius data (24.2 Å) compared to denatured states in 3.5 M guadinium chloride (28 Å) (Zurdo et al. 2001b). Our PRE and relaxation NMR studies also indicate that long-range contacts between residues with restricted motions persist in the amyloidogenic acid-unfolded state of the SH3 domain, which supports the essential role of the partly folded amyloidogenic state in amyloid formations.

Long-range interactions have also been observed in nonamyloidogenic denatured states of numerous nonamyloidogenic proteins (Schwalbe et al. 1997; Eliezer et al. 2000; Bussell and Eliezer 2001; Yao et al. 2001; Klein-Seetharaman et al. 2002; Lietzow et al. 2002; Ohnishi and Shortle 2003; Platt et al. 2005). It was proposed that native-like long-range interactions may inhibit intermolecular interactions necessary for oligomerizations (Klein-Seetharaman et al. 2002). Decreases in the extent of the long-range interactions were highly correlated with an increased amyloid-forming propensity of α-synuclein associated with Parkinson's diseases (Bertoncini et al. 2005a,b). It is somewhat contradictory to the prevailing hypothesis that partly folded amyloid precursors are prerequisite for the effective amyloid formations. Indeed extensive long-range interactions were clearly observed in our PRE study of the amyloidogenic conformational ensemble of the SH3 domain (Fig. 6). However, the long-range contacts observed in the SH3 domain appear to be mediated mainly by nonnative interactions. For example, residues in the Src loop significantly affected by the spin labeling are located far away (>20 Å) from the spin label in the native fold (Fig. 1A). The β4 strand (67–71) is also closer to residue 16 than the β3 strand (54–60) in the native state structure, although the residues in the β3 strand are more strongly influenced (Fig. 6). This indicates that nonnative long-range contacts are dominant in the amyloidogenic conformational ensemble, which might be a critical difference between the nonamyloidogenic denatured states and amyloidogenic intermediate states.

The relaxation profiles demonstrated an uneven distribution of relaxation rates. Higher R_2 values were observed mainly at residues that are located in the β-strands and RT loop in the native state (Fig. 1B). The first 10 residues in both the N and C termini are highly flexible, as typically observed in denatured states of proteins. The terminal regions (1–10 and 78–85) of the SH3 domain amyloid fibrils were shown to have low H/D exchange protection factors (Carulla et al. 2005). Although the

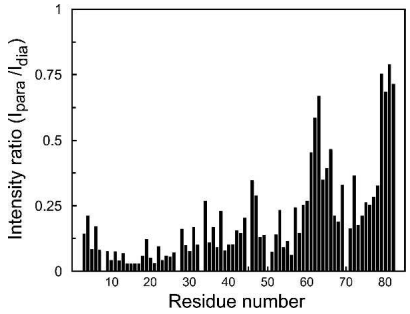

Figure 6. Paramagnetic relaxation enhancement of amide protons in the spin-labeled K16C mutant SH3 domain (pH 2.0, 35°C), which was calculated from the peak intensity ratio of the HSQC spectra in the presence (paramagnetic) and absence (diamagnetic) of the spin label.

protein studied here contained five more residues at the N terminus than previously studied SH3 domain fibrils, these additional residues did not appear to affect its amyloidogenic properties (Ventura et al. 2004). This suggests that the terminal regions are highly disordered in the amyloidogenic intermediate state and are not involved in interstrand aggregation. Additional flexible regions were identified at residues corresponding in the native protein to the β2-strand, the long Src loop, and the β4-strand. The remaining 40%–50% of the residues were found to undergo relatively restricted motion in the amyloid precursor state of the SH3 domain. The highly mobile parts of the protein might not be important for the initial intermolecular interactions necessary for the self-assembly. The correlation between the reduced mobility and the aggregation property was also observed in the reduced and oxidized β(2)-microglobulins in the intra-chain disulfide bond (Katou et al. 2002). The ^{1}H-^{15}N heteronuclear NOE experiments showed that the reduced form of β(2)-microglobulin, which is not induced to form fibrils, was more flexible than the more amyloidogenic oxidized form at pH 2.5 (Katou et al. 2002). Thus the more rigid fragments, which are mainly located in the β-strands and RT-loop, may play a key role in the effective amyloid formations of the acid-unfolded PI3K SH3 domain. This is generally consistent with recent mutagenesis studies that showed that charged residues in the RT loop and the diverging turn (17–25) play more critical roles in amyloid formation than the long flexible Src loop (Ventura et al. 2004). For example, α-spectrin SH3 domain that does not contain the long Src loop (Fig. 1) was shown to effectively form amyloid fibrils at pH 2.0 when residues 21–26 (DIDLHL) in the PI3K SH3 domain were engineered to the nonamyloidogenic SH3 domain with the wild-type sequence (Ventura et al. 2004). In addition, replacement of the long Src loop in the PI3K SH3 by that of the α-spectrin did not affect the amyloidogenic properties of the PI3K SH3 domain (Ventura et al. 2002). Moreover, the Trp-55 with a higher relaxation rate (Fig. 4) was shown be involved in aggregation process by recent tryptophan emission spectroscopy (Bader et al. 2006).

The relaxation and PRE data are also in a good agreement with the fibril structures at 25 Å resolution determined by cryo-electron microscopy (Jimenez et al. 1999), which demonstrated that the fibril could not be fit with the native fold or by a single-stranded β-sheet conformation. It was suggested that the fibrils consist of four protofilaments containing β-strands connected by extensive loops and turns. Fourier transform infrared spectroscopy (FT-IR) studies further indicated that the SH3 domain fibril core formed by the interstrand aggregations is composed of only ~40% of the protein sequence (Zurdo et al. 2001a). Thus, the 40%–50% of the more rigid residues in the amyloidogenic state may play a critical role in the interstrand aggregations, while the flexible regions remain as loops and turns in the fibril forms of the SH3 domain. It would be of great interest to investigate the correlations between the dynamical features and amyloidogenic properties of the polypeptide in more detail, which is currently underway in our laboratory.

Materials and methods

Protein samples

DNA plasmid (pGEX6P-1) for a GST-fusion PI3K SH3 domain was kindly provided by Prof. Okishio (Kanazawa University Faculty of Medicine, Japan). The GST-fusion proteins were expressed using an *Escherichia coli* BL21 strain (Novagen) and purified with GST-binding resins. The fusion proteins were subsequently digested by using PreScission cleavage enzyme (Amersham Biosciences) according to the manufacturer's recommended procedure, which resulted in an additional five residues (GPLGS) at the N terminus of the SH3 domain. The cleaved proteins were additionally purified by using HPLC with a Superdex 75 gel-filtration column (Amersham Biosciences). Concentration of the proteins was determined by measuring the UV absorbance at 280 nm in 6 M GdmCl solution with the molecular extinction coefficient of 14,650 M^{-1} cm^{-1}.

A single cysteine-containing mutant (K16C) was prepared using the GeneEditor site-directed mutagenesis kit (Promega), which was confirmed by DNA sequencing. The nitroxide spin label, MTSL [(1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl)-methanethiosulfonate; Toronto Research Chemicals Inc.], was attached to the mutated cysteine residue. The mutant SH3 domain sample was initially kept in the reduced state with 1 mM DTT, which was removed by using size exclusion chromatography (PD-10 columns, Amersham Biosciences). A 10-fold molar excess of MTSL was added to the mutant sample and incubated for 8 h at room temperature. The protein samples were extensively dialyzed to remove unreacted MTSL.

Amyloidogenic properties of the 90-residue SH3 domain were examined with electron microscopy (EM) and thioflavine T binding assays, which were identical to those of previous studies (Guijarro et al. 1998). Samples with protein concentrations >~0.4 mM (pH 2.0, 35°C) became a viscous gel after 1 wk of incubation and eventually formed highly ordered amyloid fibrils. The aggregation properties were not affected by the mutation. Under this amyloidogenic condition (pH 2.0), the SH3 domain samples (0.5–1.0 mM) were found to be predominantly monomeric over the first 40 h (Zurdo et al. 2001b). At a lower protein concentration (0.05–0.15 mM), the NMR signals of the acid-denatured states remained unchanged over a 2-wk period. This indicates that the protein exists in a stable, monomeric form at the lower protein concentrations.

NMR spectroscopy

All NMR spectra were obtained at 35°C on 500- and 800-MHz Varian Inova spectrometers equipped with a cold and room temperature NMR probe. A series of 3D experiments (HNCO, HNCACB, CBCA(CO)NH, ^{15}N-edited NOESY-HSQC, and ^{15}N-edited TOCSY-HSQC) were carried out for the backbone

assignment and secondary structure analyses. Relaxation parameters ($T_{1\rho}$, T_2, and NOE) were all obtained in an interleaved fashion to ensure identical experimental conditions for each relaxation delay. PRE effects were measured from the peak intensity ratio in the HSQC spectra of paramagnetic and diamagnetic state of the mutant (K16C) SH3 domain. The HSQC spectra for the diamagnetic state of the SH3 domain were collected from the pure ^{15}N-labeled SH3 domain as well as from the ^{15}N-labeled sample mixed with unlabeled SH3 domain attached to MTSL. The two NMR spectra were identical in terms of the signal intensity as well as the chemical shift of the NMR resonances, suggesting that intermolecular interactions are negligible under the experimental conditions.

Protein concentrations of 0.05–0.15 mM (pH 2.0) were used for all of the experiments. NMR signal intensities were measured at the beginning and end of the data collection to determine the extent, if any, of time-dependent oligomerization, and no signal changes were detected. The HSQC spectrum of the K16C mutant (pH 2.0) was identical to those of the wild-type SH3 domain except for residues 14–17, and the dynamical behavior of the SH3 domain, observed by the $R_{1\rho}$ experiment, was not changed by the mutation.

Acknowledgments

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